EFFECTS OF TORULASPORA DELBRUECKII AND METSCHNIKOWIA PULCHERRIMA ON OENOCOCCUS OENI AND MALOLACTIC FERMENTATION

Aitor Balmaseda Rubina

UNIVERSITAT ROVIRA i VIRGILI

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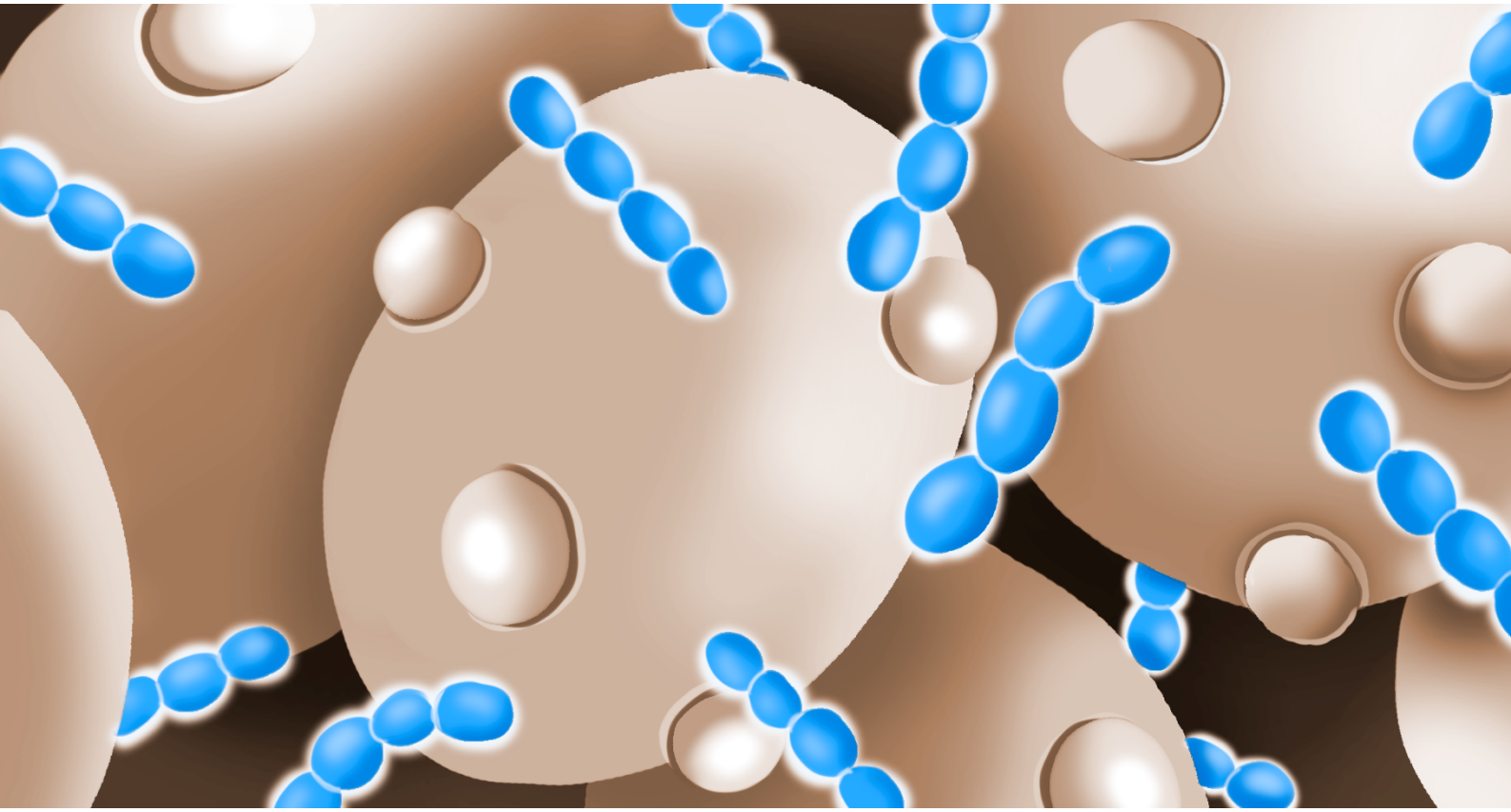
AITOR BALMASEDA RUBINA



DOCTORAL THESIS
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Aitor Balmaseda Rubina

Effects of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* on *Oenococcus oeni* and malolactic fermentation

Doctoral Thesis

Supervised by Dra. Cristina Reguant Miranda and

Dr. Albert Bordons de Porrata-Doria

Department of Biochemistry and Biotechnology

Universitat Rovira i Virgili

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UNIVERSITAT ROVIRA i VIRGILI

Dept. Bioquímica i Biotecnologia
Universitat Rovira i Virgili
C/ Marcel·lí Domingo, 1
43007 Tarragona

WE STATE that the present study, entitled "Effects of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* on *Oenococcus oeni* and malolactic fermentation", presented by Aitor Balmaseda Rubina for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of Universitat Rovira i Virgili.

This thesis is eligible to apply for the Degree of Doctor with International Mention.

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Doctoral Thesis Supervisors:

Dra. Cristina Reguant Miranda

Dr. Albert Bordons de Porrata-Doria

"El modo de dar una vez en el clavo
es dar cien veces en la herradura"

– Miguel de Unamuno

A mis padres,
nire gurasoei.

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Summary

Oenococcus oeni is the main agent responsible of wine malolactic fermentation (MLF). This lactic acid bacterium overcomes difficult and harsh wine conditions in order to finish MLF, which usually takes place after alcoholic fermentation, traditionally undergone inoculating *Saccharomyces cerevisiae*. In this sense, the current interest in non-*Saccharomyces* yeasts open a new scenario where the interactions between them and *O. oeni* are still unknown. The aim of this thesis was to evaluate the effects of non-*Saccharomyces*, particularly *Torulaspora delbrueckii* and *Metschnikowia pulcherrima,* on *O. oeni* and MLF.

Firstly, these effects were studied under real winemaking conditions, where the use of non-*Saccharomyces* reduced the MLF duration and modulated the wine organoleptic attributes. From those, polyphenolic composition was enhanced by the use of *T. delbrueckii*. Then, the performance of MLF was evaluated under lees of different yeast species, resulting an improvement in those wines with *T. delbrueckii* lees. From this, the use of mannoproteins was related with a useful metabolism of *O. oeni* to face wine conditions. The genes related with this metabolism responded to these conditions and consumption of mannoproteins was increased in wines with the highest mannoprotein content. Also, a combined omic approach was used to identify those molecular changes activated in *O. oeni* by non-*Saccharomyces*. These changes were mainly related with amino acid and carbohydrate metabolisms. Moreover, a complex metabolism of amino acids affected by non-*Saccharomyces* was observed, in which peptides are the key nitrogen compound in *O. oeni*.

The results of this thesis contribute to better understand the general impacts of non-Saccharomyces in *O. oeni* and how the bacterium respond to those changes at molecular level. In addition, this thesis points the main key elements and metabolisms to consider in the selection of suitable yeast- *O. oeni* strain tandem for winemaking.

Resumen

Oenococcus oeni es el principal microorganismo responsable de la fermentación maloláctica (FML) del vino. Esta bacteria láctica se impone a las difíciles condiciones enológicas para terminar la FML, que normalmente ocurre tras la fermentación alcohólica, tradicionalmente llevada a cabo inoculando *Saccharomyces cerevisiae*. El actual interés en las levaduras no-*Saccharomyces* abre un nuevo horizonte donde las interacciones entre ellas y *O. oeni* son todavía desconocidas. El objetivo de esta tesis fue la evaluación de los efectos de las no-*Saccharomyces*, particularmente *Torulaspora delbrueckii* y *Metschnikowia pulcherrima*, en *O. oeni* y la FML.

En primer lugar, se vio que en condiciones de bodega el uso de no-*Saccharomyces* redujo la duración de la FML y moduló los atributos organolépticos de los vinos. Entre ellos, la composición polifenólica fue potenciada por el uso de *T. delbrueckii*. Después se evaluó el comportamiento de la FML en contacto con lías de diferentes especies, obteniéndose un rendimiento superior con lías de *T. delbrueckii*. Con ello, se confirmó la utilidad de las manoproteínas en la adaptación de *O. oeni* al vino. Los genes relacionados con el metabolismo de manoproteínas se sobreexpresaron en condiciones enológicas y su consumo fue mayor en aquellos vinos con las concentraciones más altas. También se utilizó un enfoque ómico combinado para identificar los mecanismos moleculares activados en *O. oeni* por las no-*Saccharomyces*. Los principales cambios observados fueron los relacionados con el metabolismo de aminoácidos y carbohidratos. También se observó un complejo metabolismo aminoacídico, donde los péptidos jugaron un papel fundamental, afectado por las no-*Saccharomyces*.

Los resultados de esta tesis contribuyen a comprender mejor el impacto global de las no-*Saccharomyces* en *O. oeni* y cómo la bacteria responde a ellos a nivel molecular. Además, esta tesis señala los elementos y metabolismos claves a considerar en la selección de combinaciones levadura – *O. oeni* adecuadas para vinificación.

Resum

Oenococcus oeni és el principal microorganisme responsable de la fermentació malolàctica (FML) del vi. Aquest bacteri làctic s'imposa a les difícils condicions enològiques per acabar la FML, que normalment succeeix després de la fermentació alcohòlica, tradicionalment portada a terme inoculant *Saccharomyces cerevisiae*. L'interès actual en els llevats no-*Saccharomyces* obre un nou horitzó on les interaccions entre aquests i *O. oeni* són encara desconegudes. L'objectiu d'aquesta tesi va ser l'avaluació dels efectes dels no-*Saccharomyces*, concretament *Torulaspora delbrueckii* i *Metschnikowia pulcherrima*, en *O. oeni* i la FML.

En primer lloc es va comprovar que en condicions de celler l'ús de no-*Saccharomyces* va reduir la durada de la FML i va modular els atributs organolèptics dels vins. D'aquests atributs, la composició polifenòlica va ser potenciada per l'ús de *T. delbrueckii*. Després es va avaluar el comportament de la FML en contacte amb lies de diferents espècies, i es va obtenir un rendiment superior en vins amb lies de *T. delbrueckii*. Amb això es va confirmar la utilitat de les manoproteïnes en l'adaptació d'*O. oeni* al vi. Els gens relacionats amb aquest metabolisme es van sobreexpressar en condicions enològiques i el consum de manoproteïnes va augmentar en aquells vins amb concentracions més altes. També es va utilitzar un enfocament òmic combinat per identificar els mecanismes moleculars activats en *O. oeni* pels no-*Saccharomyces*. Aquests canvis es relacionaven sobretot amb el metabolisme d'aminoàcids i carbohidrats. També es va observar un complex metabolisme aminoacídic, on els pèptids van jugar un paper fonamental, afectat per les no-*Saccharomyces*.

Els resultats d'aquesta tesi contribueixen a comprendre millor l'impacte global de les no-*Saccharomyces* en *O. oeni* i com el bacteri respon a ells a nivell molecular. A més, aquesta tesi assenyala els elements i metabolismes claus a considerar en la selecció de combinacions llevat - *O. oeni* adequades per la vinificació.

1. Introduction

1.1. Introduction to wine and winemaking

Wine is one of the most important beverages of our society. This has been like this since the beginnings of the civilizations. Probably, some harvested and damaged grapes spontaneously started fermenting producing some kind of fermented beverage – wine. So, wine, apart from playing an important role in our ancestors becoming sedentary humans, it was one of the first biotechnology product. Even if our ancestors did not know what they were doing, they were transferring microorganisms into a fresh juice to mimic what spontaneously occurred before: what now we call biotechnology.

After that, we had to wait lots of centuries to unreveal the scientific secrets behind the winemaking process (Chambers and Pretorius, 2010): Antonie Lavoisier settled the bases of the alcoholic fermentation (AF) in 1789; Louis Pasteur postulated the AF as energy pathway of yeasts under anaerobic conditions in 1870; Hermann Müller-Thurgau demonstrates that bacteria are responsible of the malolactic fermentation (MLF) in 1891; Eduard Buchner study the ability of yeast extracts -enzymes- to ferment sugars despite the absence of living yeast cells in 1897, and so on.

Since then, the knowledge in wine production and its improvement increased exponentially and allowed winemakers to produce technological and good quality wines. Wines produced with knowledge and technology which our ancestor never could dream about.

1.1.1. Grape berry and fermenting must

Grapevine (*Vitis vinifera*) is a very extended fruit crop which provides berries that can be used as fresh fruit, raisins and for the production of fermented and distillate beverages. The vinification process takes place in the cellar, but all starts in the vineyard. Wine is produced with grown and ripen grape berries from *V. vinifera* cultivars. After pollination, fertilization and fruit set, the grape flower ovary develops into a fleshly berry (Coombe and McCarthy, 2000). This newly formatted berry will grow in four successive phases: (i) a rapid berry growth, (ii) a lag period, (iii) a rapid

berry growth and fruit ripening, phase, and finally, (iv) a senescence phase (Coombe and Hale, 1973).

During this first phase berries grow through cell division in the pericarp tissue and gradually those new cells will start cell enlargement. Water and organic acids, mainly L-malic acid, will start to be accumulated while sugar concentration remains low. At the end of this phase, the concentration of organic acids will be the highest and the high concentration of chlorophylls will turn it into green.

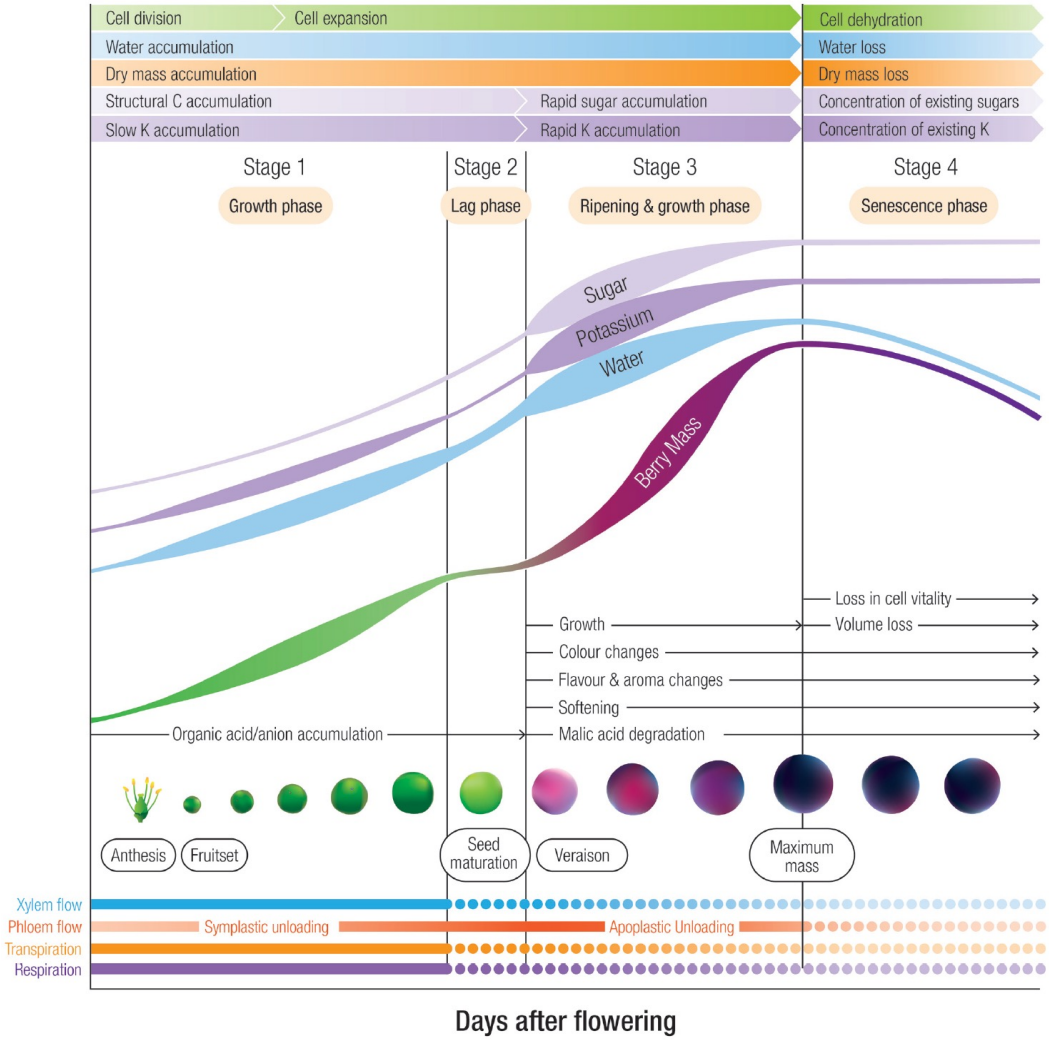

Figure 1. The four developmental stages of grape berries since flowering until senescence phase (From Rogiers et al., 2017).

One of the most dramatic changes during the second phase (lag or transition) will be the *veraison* (berry softening) which will be characterised by the colour change in the grape skin of red grape berries. This is the result of the accumulation of simple sugars, potassium and phenolic compounds, including anthocyanins in red berries

which will turn their colour from green to red. Also, berries will lose their chlorophylls and the concentration of organic acids will decrease.

In the third phase, important morphological and physiological changes will continue with highly marked hormonal changes which will drive the ripening process (Serrano et al., 2017). After, a fourth phase, commonly described as a senescence phase takes place, where the berry cells start a programmed cell death (Rogiers et al., 2017).

At the end of those three first phases, the grape berry is in optimal conditions for harvesting (Kuhn et al., 2014). These conditions include (i) high sugar concentration, (ii) relatively small number of organic acids, (iii) a juice pH between 3-4, (iv) adequate nitrogen compounds for a successful fermentation, (v) optimal polyphenolic composition, (vi) an appropriate concentration of aroma and flavour compounds and precursors, among others.

1.1.1.1. Nitrogen compounds in grapes and its role in wine fermentation

Nitrogen compounds found in grape berries are essential for successful alcoholic and malolactic fermentation. Apart from proteins and peptides, the most important nitrogen sources found in grapes are amino acids and ammonium which play an important role in yeast and lactic acid bacteria (LAB) metabolisms (Bell and Henschke, 2005). Total nitrogen concentration content increases during ripening and may decrease when grape is completely ripened (Bell and Henschke, 2005; Hilbert et al., 2003; Pandey et al., 1974). While amino acid concentration increases during ripening, ammonium slowly decreases its concentration until harvesting (Bell and Robson, 1999). It has to be mentioned that the relative abundance of each amino acid will be dependent on the cultivar, *terroir* and agricultural practices.

Grape must usually has from moderate to high nitrogen composition (0.1-1 N g/L). These includes (i) ammonium (3-10%), (ii) amino acids (25-30%), (iii) polypeptides (25-40%) and (iv) proteins (5-10%) (Ribéreau-Gayon et al., 2006). Nitrogen concentration of grapes (and, consequently grape must) specially ammonium and amino acids highly depend on the *V. vinifera* cultivar and its vigour (Van Leeuwen et al., 2000) and fertilization treatment and dosage (Hannam et al., 2016; Van Leeuwen et al., 2000). Besides, there is also a strong influence of the vintage (Hannam et al.,

2016) and edaphic properties of the soil (Pérez-Álvarez et al., 2019). As key nutrient of yeast metabolism, nitrogen concentration has to be controlled and be always enough for a good AF performance. Nevertheless, high concentration of nitrogen can promote the infection of plant pathogen as *Botrytis cinerea* (Choné et al., 2006).

At this point we should make a difference between amino acids. Taking as references the wine yeast *S. cerevisiae*, amino acids are classified into those which can be metabolized and not. *S. cerevisiae* uses as preference nitrogen sources ammonium and free alpha amino compounds (Roca-Mesa et al., 2020) which are known as yeast-assimilable-nitrogen (YAN). The remaining nitrogen compounds including secondary amino acids –proline and hydroxyproline-, polypeptides and proteins will not be assimilated by yeasts and will remain constant during the winemaking process. In this sense, the concentration of ammonium can range between 2 to 54% (Huang and Ough, 1989) which means that ammonium can represent the half of YAN concentration. Proline concentration represents more than 50% of the total amino acids and can represent up to 80% when the total amino acid concentration is low (Hannam et al., 2016). This can be particularly problematic in musts of white grapes where nitrogen concentration is very low (Dubourdieu et al., 1986), as high concentration is located in grape skins (Ribéreau-Gayon et al., 2006).

Another microbial group, which also needs a nitrogen source for developing are LAB. This group of bacteria exhibit a low demand of nitrogen in wine (Remize et al., 2005), as a consequence of the depleted medium where they must develop. They preferred nitrogen source are peptides (Remize et al., 2006), and they are more related with yeast metabolism rather than with grape must.

1.1.1.2. Polyphenolic compounds in grapes

Phenolic compounds are a chemical family of molecules which are responsible for the main organoleptic properties of red wines, such as colour, mouthfeel, astringency and bitterness (Kennedy, 2008). They come from different parts of the grape berry and they are extracted during the fermentation and maceration of the fermenting must (Ribéreau-Gayon et al., 2006). Moreover, their concentration will depend on the cultivar, ripening and the winemaking process. This family is characterized by a common chemical structure: a benzyl core with one or more hydroxyl groups (-OH)

(Monagas et al., 2005). Considering their carbon skeleton, they are divided as flavonoid and non-flavonoid. Non-flavonoids are mainly found in the flesh and flavonoids in seeds and skins (Monagas et al., 2005). The family of flavonoid phenolic compounds is much divided into tannins and anthocyanins which are the most important polyphenols in wine. Flavonoids respond to an important biological role of radiation damage and pathogen-against protection (Iwashina, 2003; Middleton, 1996; Panche et al., 2016).

Anthocyanins are responsible for the red colour of grapes and wines. They are located in the vacuoles of grape skin cells. Depending on the cultivar, the anthocyanin concentration in red grape skins is 500-3000 mg/kg (Ferrandino et al., 2012; Theodorou et al., 2019). The first anthocyanins of the grape berry start to appear about three weeks before the *veraison*, but it is really after this one that their concentrations increase significantly until maturity (Ribéreau-Gayon et al., 2006). Anthocyanins are unstable molecules and the colour they will present in solution will mainly depend on the pH, among other parameters such as SO_2. These molecules can be found in 4 different forms (Glories, 1984): (i) in flavylium cation A^+ form (red colour), (ii) in the quinone bases AO form (blue-purple colour), (iii) in the carbinols base AOH form (colourless) or (iv) in the cis and trans chalcones C form (light yellow in colour). In wine pH (around 3-4) all four forms coexist being the colourless forms the dominants, representing the 65-85% of the total anthocyanin concentration. Red colour form represents between 4 and 35% of the anthocyanins over the same pH range. Blue-purple colour form also represent a small proportion between 8 and 15% of total anthocyanins (Glories, 1984). So, depending in the pH, the colour will be altered. This is of particular interest when we think about fermentation process where AF reduces pH and MLF increases it (Liu, 2002).

1.1.2. From must to wine: biotransformation process

Grape must is different according to the winemaking process they will suffer: white or red winemaking. In the first one, grape skins and seeds will be removed to start the fermentation. On the contrary, red winemaking will need their presence during almost the whole process.

Grape must is high density (around 1080-1120 g/L), acidic (pH 3-4) and sweet raw material to work with (Ribéreau-Gayon et al., 2006). It has dissolved those compounds present in grape berries previously presented and others like microorganisms coming from the grape skins.

This liquid is going to be transformed into wine, a hydro alcoholic solution with highly changed organoleptic profile: wine. It is the consequence of the growth of yeasts, which transform simple sugars of the must into alcohol and CO_2. Of course, apart from alcohol, other elements from the grape, and also produced during AF by yeasts, will be also present in wine: amino acids, polyphenols or organic acids. Some of these compounds will be transformed by yeasts into volatile metabolites which will directly impact the aroma and flavour of wine (Pretorius, 2017; Swiegers et al., 2005).

Among other wine yeasts, *Saccharomyces cerevisiae* is the most important yeast involved in the winemaking process. It is the best adapted yeast to fermentation in cellar conditions due to its high tolerance to low pH, high osmotic pressure and high ethanol concentrations (García-Ríos and Guillamón, 2019). Nevertheless, every ecosystem is full of biodiversity and grape must is not an exception. Grape berries present high cell populations including yeasts, bacteria and moulds (Belda et al., 2017c).

Those other yeasts different from *S. cerevisiae* are metabolically active in the first stages of AF where they contribute to wine fingerprint (Petruzzi et al., 2017). Later, when the alcohol content and other stressful factors begin to appear, this other yeast begin to die while *S. cerevisiae* become dominant (Albergaria and Arneborg, 2016; Beltran et al., 2002). This group of wine yeasts are known as non-*Saccharomyces* yeasts.

Also, those other microorganisms present in the fermenting must during winemaking will have its impact in wine quality. Special attention has to be paid to LAB which are responsible of the MLF an important biotransformation in red wines and high acidity white wines (Lerm et al., 2010).

Moreover, even if they do not directly participate in the winemaking biotransformation, all the remaining microbial communities will interact and contribute somehow in final wine quality (Pinto et al., 2014).

1.1.2.1. White and red winemaking

White winemaking (Figure 2) starts with the destemming: separation of the grape berries of the bunch from the stem. Ones berries are free, the crushing and pressing of white grapes is performed to obtain the grape must, which will be fermented. Occasionally, some red grapes are also used for white winemaking. The objective of the crushing is to liberate the content of the vacuoles of the pulp which store the mayor content of sugars and acids of the grape berry cells. However, polyphenols and volatile substances still remain in grape skin, so a step of skin contact prior to pressing is usually needed to extract them. It is also possible to produce white wine with red grapes, just controlling that step of skin maceration to not extract to many polyphenols. The resulting must is a high turbid and density liquid with lots of grape solids which is clarified. Usually is kept for 24h at 4 ºC for clarification, but there exist lots of formulas for this. Clarification is important to obtain a clearer liquid to ferment with less sediments that could visually negatively impact in the costumer. During clarification must is exposed to oxidation and, furthermore, the autochthonous microbiota can spontaneously start an undesirable AF. For this purpose, sulphur dioxide is added. Concentrations around 50 mg/L of sulphur dioxide will have a protective antioxidant, antioxidasic and antimicrobial effect.

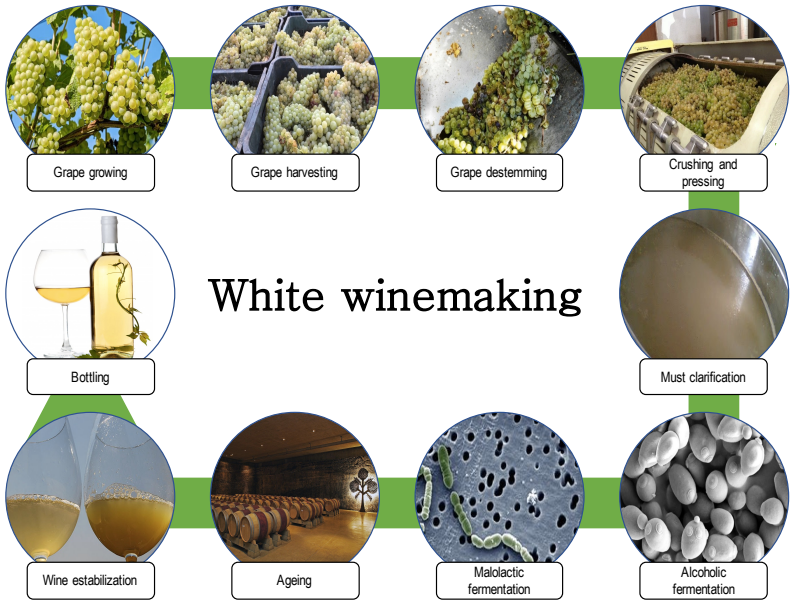

Figure 2. Main steps of white vinification process (Adapted from Jolly et al., 2014)

After clarification, white must is ready to be fermented either spontaneously with the autochthonous yeasts or by some selected yeast for the performance of AF.

The main characteristic of red winemaking (Figure 3) is the extraction of the polyphenols of grape skins. This means that grape skins will be present during more time than in white winemaking where grape berries are rapidly crushed and pressed and the skins are removed. Red grapes are destemmed, and a small scratch is made. After that, scratched grape berries are directly introduced into the fermentation tanks. In this process, grape berries are fermented as a whole with skins, pulp and seeds. During winemaking compounds contained in grape skins are released thanks to alcoholic extraction. Polyphenols and aromas are transferred from skins to fermenting must. As result of the generated CO_2 during fermentation, solids, mainly grape skins are displaced to the top of the fermenter. That combination of solids is called cap. With the purpose of optimising the extraction of skin compounds some technological operations are usually done to move and break the cap. The preferred operation is pumping over which involves the circulation of the fermenting must from the bottom of the tank over the cap of skins in the top of the tank. This provides a percolation effect where the must passes through the skins and homogenises the liquid. Other operations involve plunging or punching down, heading down boards, etc. Apart from the extraction it is important to break the cap because it accumulates a lot of heat which eventually could be inconvenient for the quality of the wine.

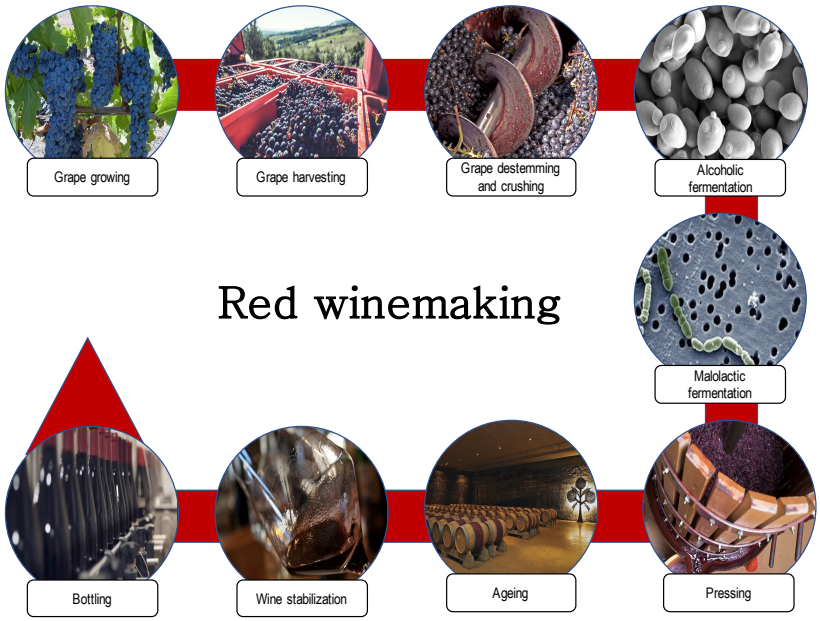

Figure 3. Main steps of red vinification process (Adapted from Jolly et al., 2014)

After AF, wine liquid is extracted from the tank. Moreover, high content of wine remains inside the skins. So, a pressing operation is needed to extract that liquid.

Once the AF of white and red wine is finished, they can undergo the MLF by LAB. And finally, some finning operations are performed to prepare the wine for consumption. Usually, MLF is performed in presence of yeast lees. Yeast lees are the fermenting yeast that after completing the AF begin to die (Belda et al., 2016). During this process yeast dead cells start to autolysate due to the hydro alcoholic environment. These releases their intracellular components and enrich wine for the growth of LAB (Diez et al., 2010).

1.1.3. Wine attributes

1.1.3.1. Volatile compounds and aromas in wine

Aroma is one of the most important sensory attributes of wine. It is compound by a pool of hundred odorant molecules in concentrations of mg/L or even ng/L. Wine aromas are generally classified in three groups: (i) varietal aromas, those coming directly from the grape berry, (ii) fermentative aromas, or secondary aromas, related to microbe metabolism and (iii) aging aromas from the aging process (Figure 4).

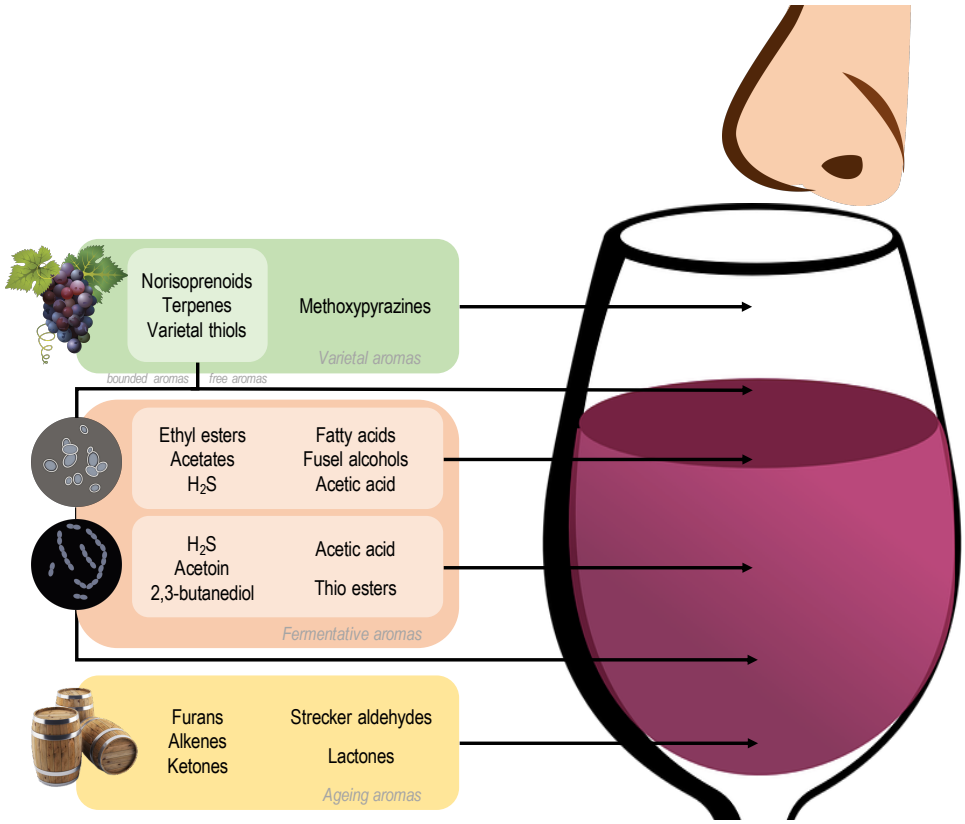

Figure 4. Wine aroma classification. Main odorant family compounds are grouped regarding to their origin.

Varietal aromas play an important role in the wine aroma. Besides, they contribute to wine quality and character more than other family aroma (Ribéreau-Gayon et al., 2006). It is determined by the *V. vinifera* cultivar of the fermenting grapes (Ruiz et al., 2019). From that high amount of odorant molecules, few participate really in the aromatic fingerprint of the cultivar (González Álvarez et al., 2011).

This family is directly present in grape must as free odorant compounds. However, the most of them are hired odorant compounds in precursor aroma forms. These compounds are generally bounded to a sugar or amino acid molecule which are not odorant. As result of chemical or enzymatic reactions during winemaking, those bounds are broken and the aroma is released as free odorant molecule (Belda et al., 2017b; Styger et al., 2011). Moreover, it exists an evolution of each aroma during the ripening process where the aroma is transformed between the odorant and non-odorant forms (Arias-Pérez et al., 2020). Also, they can be released as consequence of mastication and salivation (Ferreira and Lopez, 2019).

Varietal aromas are secondary metabolites synthesized by the vine. The main compounds of this family are terpenes and norisoprenoids (Figure 4). Also, methoxypyrazines and varietal thiols contribute significantly to these aromatic group (Ribéreau-Gayon et al., 2006).

Terpenes are the most studied varietal aromas of grape berries (González-Barreiro et al., 2015) and they exhibit the classical aroma of Muscat grapes. They can be presented as free odorant form or non-odorant volatile precursor. These precursors are mainly glycosylated. As result of the ß-glycosidase enzymatic activity of grape enzymes or microbial metabolism, they are transformed to their free odorant form (Michlmayr et al., 2012; Ribéreau-Gayon et al., 2006). The most important terpene is linalool, together with genariol, among others (Ferreira and Lopez, 2019).

Norisoprenoids come from the chemical or enzymatic breakdown of ß-carotenoids and lutein and are related with floral and fruity aromas (Gutiérrez-Gamboa et al., 2018). The most important norisoprenoids are: ß-damascenona, 1,1,6-trimethyl-1,2-dihidronaftalene (TDN), el vitispirane y la ß-ionona (Mendes-Pinto, 2009). They are mainly responsible of the varietal aroma of some cultivars as Chardonnay and Sauvignon Blanc (González-Barreiro et al., 2015).

The focus will be pointed in fermentative volatile compounds as they are the result of the metabolism of yeast and LAB during the winemaking process. These volatile compounds such as fusel alcohols, acetates, fatty acids (FA), esters, etc. (Figure 3) will be discussed latter when talking about the fermentative processes (AF and MLF).

Ageing aromas are produced during wine ageing and storage as consequence of chemical oxidations and aroma transference from the oak barrel. The main family compounds related with ageing aromas are furans, Strecker aldehydes, alkenes, ketones and lactones (Mayr et al., 2015). They are associated with olfactory sensations of woody, balsamic, confectionery fruit, etc. (Escudero et al., 2000; Silva Ferreira et al., 2003). Besides, they play an important role on the aroma quality of wines from overripe grapes as Pedro Ximenez or Sauterness (Issa-Issa et al., 2020). Usually, their concentration during wine ageing increase while varietal and fermentative aromas decreases (Dumitriu et al., 2019; Loscos et al., 2010). This evolution is due to oxidation reactions. Indeed, (Mislata et al., 2020) observed that Rioja red wines after strong oxidation, exhibited a loss of 40% on the fermentative aromas and an increase of 85% on the ageing aromas.

1.1.3.2. Wine polyphenols

As wine colour is the result of the present colourful forms, we need to precisely quantify and catalogue the colour of red wines. For this purpose, CIELAB colorimetry model is used to describe wine colour. The CIELAB model, adopted by the CIE (International Commission on Illumination) in 1976. It represents a complex colour space which models human vision and is independent of any material. The CIELAB model builds on the theory of opponents by drawing inspiration from Richard Hunter's former Model Lab (1942). The representation of a wine sample in the CIELAB space depends on its coordinates: lightness (L*), chroma (C*), hue (h*), red–greenness (a*), and yellow–blueness(b*) which precisely define the colour of the sample.

Apart from their contribution to wine colour, they seem to have no other flavour properties in the concentrations found in wine (Castañeda-Ovando et al., 2009). 15 anthocyanins can be found in wines produced with *Vitis vinifera* grape berries where malvidin-3-*O*-glucoside is the most abundant one (Ribéreau-Gayon et al., 2006)

Tannins are a group of molecules that can form stable bounds with proteins (Lamy et al., 2011), polysaccharides (Li et al., 2019) and alkaloids. Generally, tannins are classified as hydrolysable tannins and non-hydrolysable tannins according to Freudenberg since 1920. This classification has been recently updated defining non-hydrolysable tannins as condensed tannins or proanthocyanidins (Smeriglio et al., 2017). They are responsible for the astringency of wine, that is, the sensation of dryness and roughness in the mouth epithelium caused by astringent factors as tannins (Llaudy et al., 2004). Red wine astringency is the result of the interaction of wine tannins with salivary proteins though the formation of hydrophobic complexes or hydrogen-bonded cross-linked complexes. Altogether, tannins contribute to the organoleptic profile of wines as astringency is one of the most important properties which define the quality of red wines. (Gawel, 1998; Gawel et al., 2000).

1.1.3.3. Perception of sensory attributes of wine

Wine chemical composition is a complex matrix to evaluate (Cayot, 2007). There are hundreds of molecules which have some sensory effect in the costumers, in what is called *flavour*. The flavour is defined as the "perception resulting from stimulating a combination of the taste buds, the olfactory organs, and chemesthetic receptors within the oral cavity" (Waterhouse et al., 2016). In this sense, the concentration of those molecules is extremely low. Apart from water, ethanol, glycerol, tartaric and malic acids, the rest of the molecules are in concentrations of mg/L, ng/L or even pg/L (Figure 5). Those molecules interact and can exhibit synergic or antagonistic effects which result in higher or lower perception in the costumer.

The perception of a molecule depends on the threshold concentration (Francis and Newton, 2005). It determines the minimum quantity of the substance that can be detected by the person. It can be affected by the sex of the person, the expertise and the interaction with other molecules.

Wine tasting is the tool to assess the quality and the impact of particular winemaking practices in the wine organoleptic profile. It is important since chemical modification are not always precepted by the costumers. Wine tasting is composed by three phases: (i) visual phase, where the colour and visual appearance of wines is tested; (ii) olfactory phase, where the volatile composition is analysed and (iii) tasting where

the tactile attributes are studied. The summary of these three phases can assess the impact of the chemical composition in the *flavour* exerted by the product and, consequently precepted by the consumer.

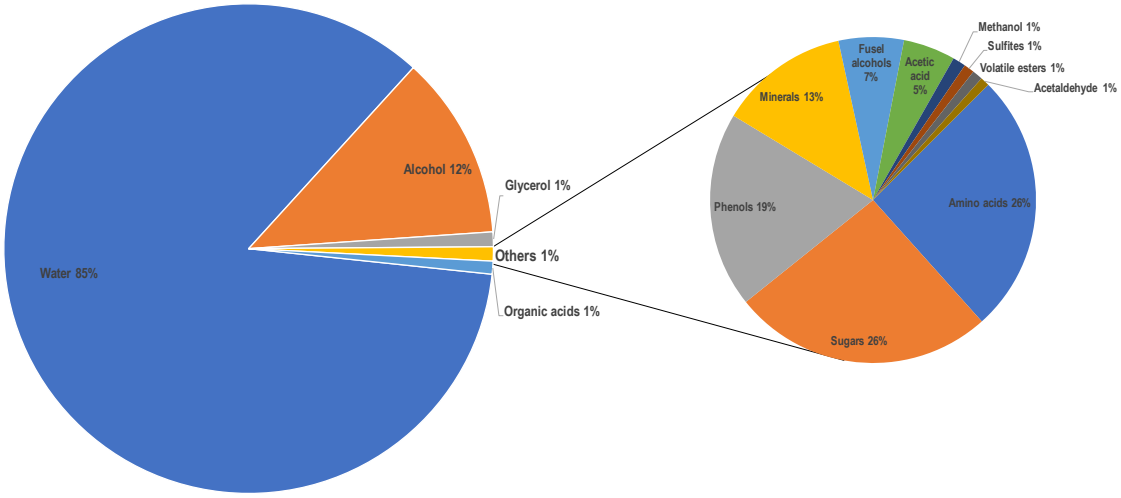

Figure 5. Representative composition (% w/w) of an average dry red table wine (Adapted from Waterhouse et al., 2006).

1.1.4. Climate change in winemaking

In the last decades the temperature of the Earth is increasing (Ubeda et al., 2020). One of the most affected sectors is the agricultural sector which is losing their cultivable lands as consequence of desertification. Indeed, wine industry is affected since it depends on grape berry development. The main consequence is that the grape berry accumulates more sugars and has lower acidity.

As temperature rises, the sugar accumulation in grape is faster so the optimal ratio sugar:acids is earlier achieved. It is not the same for the development of secondary products as polyphenols and aromas. So, the higher the temperature, the more decoupled are these two ripening processes (Pons et al., 2017).

In this new challenging scenario, winemakers have to harvest grapes earlier to their ideal accumulation of secondary products to ensure an adequate sugar content to not have fermentation problems (Fraga, 2019; Hannah et al., 2013). Besides, high alcoholic content wines are not desirable by costumers.

As consequence, the extraction of secondary metabolites, mainly from grape skins, has never have such an importance. As well as the development of strategies form reducing ethanol in winemaking. It is now necessary to have high extraction yields to overcome the lees concentration of flavours and low ethanol yields. In this way, the use of microbial resources, such as non-conventional yeasts can be a particular answer to these problems by lowering ethanol (Contreras et al., 2014; Quiros et al., 2014; Zhu et al., 2020), modulating wine acidity (Vilela, 2019) and extracting flavours (Escribano-Viana et al., 2019).

1.2. Alcoholic fermentation and wine yeasts

The most important step of winemaking is the AF. As previously introduced the changes occurring in this step are crucial for the obtaining of wine. This biotransformation is performed in grape must where a complex microbiota is present (Bagheri et al., 2020). Considering the main microbial groups of oenological interest in the grape berry, fermentative yeasts are the predominant (10^4-10^6 CFU/g) followed by LAB (10^3 CFU/g) and acetic bacteria (10^2-10^3 CFU/g) (Bae et al., 2006). These population and proportions can be affected by the health status of the grape (Barata et al., 2012).

From those, a diverse group of different yeast genera and species are the main AF agents. These yeasts coming from the mature harvested grapes have to be adapted to the changing environment found in fermenting must (Beltran et al., 2002). What is more, some other yeasts that can be found in the fermenting may not come from the grape but from the winery (resident microbiota). Most of them belong to non-*Saccharomyces* species and it is not until middle stages of AF when *S. cerevisiae* will appear (Beltran et al., 2002). High sugar concentration, which results in high osmotic pressure, and low pH of grape must will be responsible of the selection of those yeast species that will ferment the must (García-Ríos and Guillamón, 2019; Pizarro et al., 2007). Other compounds can also participate in the selective pressure of the must such as the addition of sulphur dioxide, or the low concentration of nitrogen compounds (García-Ríos and Guillamón, 2019). Since yeasts rapidly consume sugars and transform it to ethanol and CO_2, the selective pressures change and the fermenting

medium develop to anaerobic (Garijo et al., 2011). Also, the quick metabolism of yeasts impoverishes the must in terms of nutrients in high speed.

Species from the genera *Hanseniaspora*, *Pichia*, *Candida, Kluyveromyces* and *Metschnikowia* are found in that early stage of AF. Occasionally, *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Dekkera* and *Schizosaccharomyces* species can be found (Fleet, 2003).

During the first days this yeast community start to grow until they reach 10^6-10^7 CFU/mL. As the ecosystem begin to change, their viability starts to decrease and the previously not detected *S. cerevisiae* starts to grow (Beltran et al., 2002; Fleet, 2008). By mid-fermentation the growth of *S. cerevisiae* is the dominant one of the fermenting must reaching populations of 10^7-10^8 CFU/mL (Albergaria and Arneborg, 2016). At this point the fermentation is only controlled by *S. cerevisiae* and it ensures a successful complete consumption of sugars. However, depending on the grape health status and the winemaking operations the imposition of *S. cerevisiae* can undergo difficulties which can occasionally lead to stuck or sluggish fermentations. Besides, the complex microbiota interactions present during AF, mainly the first stages, will determine the outcome of the AF (Bagheri et al., 2020).

1.2.1. Yeast metabolism in wine

Their main metabolism is sugar metabolism related to the AF. Still, other metabolisms are important for their survival as nitrogen and fatty acids (FA) metabolism. Apart from the role in yeast growth, these metabolisms are responsible for the production of many products of oenological interest found in wine.

1.2.1.1. Sugar metabolism: glycolysis, alcoholic fermentation, glyceropyruvic fermentation and Krebs cycle

Hexoses (glucose and fructose) are the main sugar substrates found in grape must (Figure 6). That is why, these molecules are the preferential carbon source for yeast catabolism in wine. The transport into the cell is driven by membrane-bound transporters encoded in several genes (Diderich et al., 1999).

Glycolysis is a ubiquitous pathway present in yeasts (Barnett and Entian, 2005). It involves the process where a hexose is oxidized to pyruvate. During the process, NAD^+ is reduced to NADH and two molecules of ATP are produced. Steps from glucose to pyruvate are commonly known as Embden-Meyerhof-Parnas (EMP) pathway.

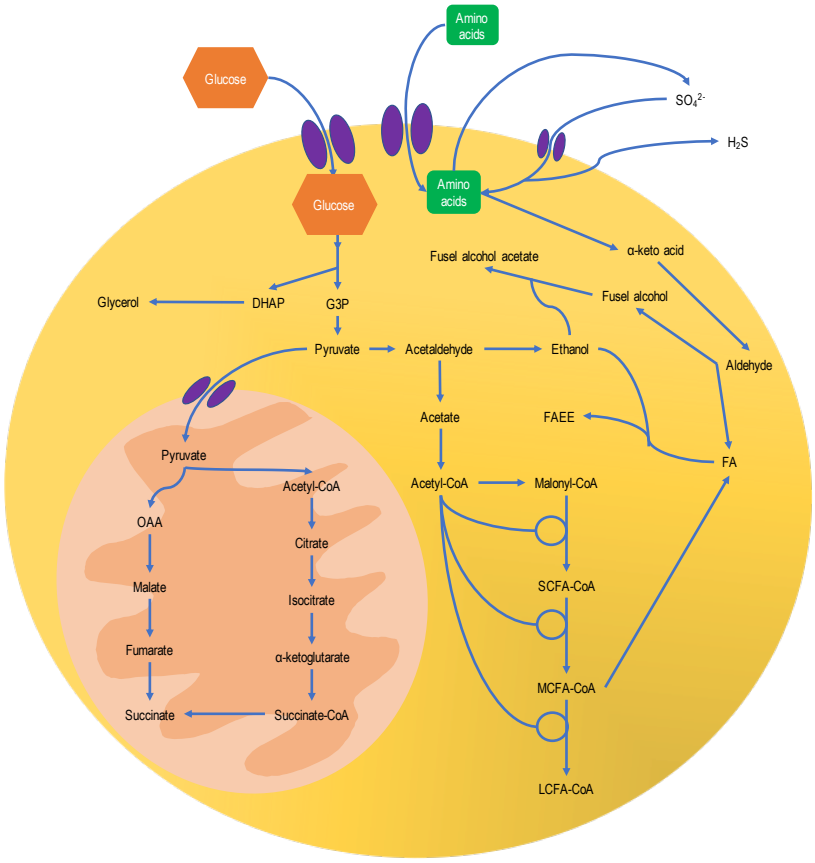

Figure 6. Scheme of the most relevant oenological metabolisms of yeasts. CoA: coenzyme A, DHAP: Dihydroxyacetone phosphate, FA: fatty acids, FAEE: fatty acid ethyl esters, G3P: Glyceraldehyde 3-phosphate, OAA: oxalacetate.

Under fermentation conditions, those reduced NADH are accumulated due to the high hexose concentration and, consequently high activity of this pathway. That is why pyruvate is used to re-establish the redox balance between NADH and NAD^+. Pyruvate is cleaved into acetaldehyde and CO_2 by the pyruvate decarboxylase (*PDC1, PDC5* and *PDC6*). Then, acetaldehyde is reduced to ethanol by alcohol dehydrogenase (*ADH1, ADH2, ADH3, ADH4* and *ADH5*) or be metabolized to cytosolic acetyl-CoA via acetate by acetaldehyde dehydrogenase and acetyl-CoA synthase. This acetyl-CoA is used for the biosynthesis of lipids and amino acids.

Another pathway yeast can use to re-establish the redox balance is the glyceropyruvic fermentation. An intermediate of glycolysis, through glycerol 3-

phosphate dehydrogenase is converted to glycerol-3-phosphate, which is an intermediate to glycerol. Finally, glycerol 3-phosphatase produces glycerol as end product of this fermentation. This pathway is usually active in the first stages of the AF when alcohol dehydrogenase lacks (Flores et al., 2000; Ribéreau-Gayon et al., 2006). Indeed, the production of glycerol helps the yeast to face the osmotic pressure found in grape must (García-Ríos and Guillamón, 2019).

The citric acid cycle (TCA) or Krebs involve 8 enzymatic reactions which completely oxidize acetyl-CoA into CO_2 and H_2O (Ribéreau-Gayon et al., 2006). These cycle takes place in the mitochondrion, in contrast to the other previous pathways driven out in the cytosol. In anaerobic conditions where oxygen is present the main carbon flow passes through this cycle generating high reductive power which is later used to produce energy in the electron transport chain (ETC). Pyruvate is transported into the mitochondria. Then, pyruvate is oxidized to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex, resulting in one molecule of $NADH+H^+$. Once acetyl-CoA is formed, enters TCA cycle by combining with citric acid by citrate synthase.

However, under fermentation conditions, several enzymes in the Krebs cycle have minimal activity, most notably citrate lyase and succinic dehydrogenase. As a result, the Krebs cycle acts like two independent branches under fermentation conditions (Waterhouse et al., 2016). Even if this uncoupled TCA cycle dos does not act as energy pathway under fermentation conditions, it represents an important biosynthetic pathway for some intermediary metabolites.

Succinic acid is the TCA cycle's most generated metabolite. It is mainly formed by the reductive branch from pyruvate (Camarasa et al., 2003). Also, succinic acid concentration can increase if the yeast uses amino acids (as aspartate, GABA and glutamate), to produce oxaloacetate and then, succinate. In this process, $NADH+H^+$ is produced which contrasts with the need to re-oxidize this cofactor in fermentation condition. However, FAD^+ is also obtained which is essential for FA biosynthesis. It is not usually to produced malic acid or citric acid, since their concentration is high in grape must and they can be incorporated from it. So, it is common to have little loss of those after AF.

1.2.1.2. Fatty acids and ethyl esters

Biosynthesis of fatty acids (Figure 6) is a very important metabolism in yeast since FA constitute the basic component of lipids. Depending on the double bound content they are classified as (i) saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) (Waterhouse et al., 2016).

Fatty acids play an important role in cellular membrane and, thus, in the response to environmental stresses (Hunter and Gaston, 1988; Nielsen, 2009). Also, they participate in cell secretion and can be used as energy source through ß-oxidation pathway (Jump, 2004; Van Meer et al., 2009; Trotter, 2001).

Fatty acids are synthesised in the cytosol from acetyl-CoA (Waterhouse et al., 2016). Also, fatty acids can be synthesised in the mitochondria (Tehlivets et al., 2007). This acetyl-CoA is formed in the cytosol, contrary to the one used in the TCA cycle. It is formed from acetic acid coming from the oxidation of acetaldehyde. The reactions of this pathway are catalysed by the multienzyme fatty acid synthase complex (FAS). The pathway begins with the elongation of acetyl-CoA to malonyl-CoA to form a 4-carbon (C4) intermediate. The, further elongation steps follow with the combination of more molecules of malonyl-CoA to produce intermediates of C6, C8 and so on skeleton FA, before eventual release of the saturated palmitate FA (C16:0). Branched-chain and odd-numbered FA can be formed through the same pathway by substituting the acetyl-CoA for another acyl-CoA (Martin and Vagelos, 1960). FA with an odd number of carbons can be formed starting from propionyl-CoA (Luttik et al., 2000). Apart from those long chain FA, less long chain FA are produced during the metabolism of yeast in AF.

FAs are also classified regarding to their chain length as: short chain FA (SCFA), C3-C5; medium chain FA (MCFA), C6-C12 and long chain FA (LCFA), >C12. LCFA are responsible of the integrity of the cell membrane, but they do not participate in the organoleptic properties of wine. However, less long chain FA, as they are more volatile, they directly impact in the volatile composition of wines.

Acetic acid is formed as a metabolic intermediate in the synthesis of acetyl-CoA from pyruvic acid. It is formed directly from acetaldehyde by aldehyde dehydrogenases. MCFA accumulation result in the inhibition of the first stages of FA

synthesis (Saerens et al., 2010) and they are released from the cell and, consequently, accumulated in wine.

FA can eventually form ethyl esters in alcoholic media through esterification. This reaction can occur via enzymatic catalysis or during ageing by non-enzymatic acid chemical reaction. The reaction is in equilibrium between the free FA and its FA ethyl ester (FAEE). Mainly, MCFA produce FAEE and have oenological interest (Francis and Newton, 2005). In the enzymatic catalysis two acyl-CoA:ethanol *O*-acyltransferase enzymes (*EHT1, EEB1*) are involved (Saerens et al., 2006). Still, the production of FAEE is very complicated and is highly dependent on oxygen availability (Mason and Dufour, 2000). For the enzymatic esterification the FA is activated with CoA and the resulting acyl-CoA is condensed with ethanol. Enzymatic catalysis has low performance so the majority of the FAEE are going to be produced by chemical condensation in wine matrix. Besides, the expected FA:FAEE molar ratio in wine matrix is 6:1 (Waterhouse et al., 2016).

FAEE contribute to desirable aromas related with fruity sensations, candy and perfume-like aromas which contribute to freshness character of wines (Pires et al., 2014; Ribéreau-Gayon et al., 2006; Takahashi et al., 2014). That is why winemakers are interested in increasing the concentration of MCFA to promote the chemical formation of the FAEE.

1.2.1.3. Nitrogen metabolism, fusel alcohols, acetates and sulphur compounds

The uptake of amino acids and ammonium is highly regulated by the nitrogen catabolic repression (NCR) of yeasts (Beltran et al., 2004; Lleixà et al., 2019; Roca-Mesa et al., 2020) which controls the expression and degradation of amino acid permeases. In the first AF stages when nitrogen is abundant, the NCR represses the uptake of non-preferential nitrogen sources, different from ammonium, glutamine or asparagine. Then, a de-repression of the NCR is performed to allow the entrance of other nitrogen sources (Beltran et al., 2004).

So, the control and measurement of YAN prior to fermentation is crucial to prevent fermentation problems and ensure a complex volatile composition of wine. With that purpose diammonium phosphate has been used for supplementing nitrogen in musts.

Currently it is usually substituted by diammonium sulphate (Ribéreau-Gayon et al., 2006). Strong inhibition of AF is observed when YAN is below 140 mg N/L (Beltran et al., 2004; Lleixà et al., 2019) or ammonium concentration is less than 25 mg/L (Ribéreau-Gayon et al., 2006). In those cases, the supplementation with an ammonium salt is necessary.

In addition, amino acids play an important role in the production of fusel alcohols as consequence of yeast metabolism. Some amino acids (branched-chain amino acids -isoleucine, leucine and valine-, aromatic amino acids -phenylalanine, tyrosine and tryptophan- and the sulfur-containing amino acid -methionine-) are catabolised by the Ehrlich pathway and can produce fusel alcohols (specially 2-phenylethanol) depending on the redox state of the cell (Mas et al., 2014). Besides, the regulation of the Ehrlich pathway will depend on the yeast species and the carbon and nitrogen compounds availably (González et al., 2018b; Lacroux et al., 2008).

Also, fusel alcohols can be substrate for the formation of acetate esters by enzymatic acetylation by acetyl-CoA. This reaction is catalysed by acetyltransferase enzymes (*ATF1, ATF2*). This group of molecules are related with fruity and floral descriptor which contribute to the freshness character of young wines. The percentage of the fusel alcohols transformed to acetate ester is very low (Ugliano and Moio, 2005) and it is controlled by the expression of the acetyltransferase enzymes not by the substrate concentration.

Another product that can be formed as consequence of nitrogen metabolism is hydrogen sulphide (H_2S). H_2S is a common metabolite of yeast metabolism (Kumar et al., 2010). The S containing amino acids and nucleosides need SO_4^{2-} usually taken from the media (Waterhouse et al., 2016). In the biosynthesis S^{2-} can be generated in more concentration than needed for the pathway. If it is not incorporated to the amino acid or nucleoside, it is released as H_2S. So, the management of YAN in grape must is important to avoid the need of using this biosynthetic pathway. In this sense, when nitrogen precursors are depleted, higher concentrations of H_2S is found in wine (Jiranek et al., 1995). Also, sulphur compounds can be generated from the degradation of sulfurated amino acids, cysteine and glutathione (Robinson et al., 2014).

The production of H_2S depends on the yeast strain and the composition of the media in terms of nitrogen (Ugliano et al., 2011) and micronutrients (Kumar et al., 2010) However, it is difficult to assess the relation with the produced H_2S and the concentration in wine, mainly due to the influence of other chemical compounds found in wine (Bekker et al., 2016; Ugliano et al., 2011).

H_2S is of particular interest because it is related to undesirable aroma descriptors. H_2S is an important contributor to the reductive off-flavour on wines often described as rotten eggs or putrefaction.

1.2.2. *Saccharomyces cerevisiae*

S. cerevisiae is the most important wine yeast species involved in winemaking process (Ribéreau-Gayon et al., 2006). It is the one that is desirable to carry out the AF in the final stages because its high fermentative capacity. This yeast can consume nearly all the sugar present in the fermenting must before it loses viability. This is a particularly important capacity because other yeasts eventually start dying when ethanol and SO_2 concentrations are high. Moreover, it is metabolically very active even under physicochemical conditions present in wine fermentation condition like low pH and nutrient and oxygen concentration. *S. cerevisiae* is capable of fermenting sugars into ethanol in a very high yield which also contribute to control the autochthonous microbiota since *S. cerevisiae* itself present high ethanol tolerance.

This is thanks to the Crabtree effect which allows this particular yeast to consume sugars in high rate and produce ethanol when fermenting in high sugar concentrations as it is found in winemaking, even in presence of oxygen (Barnett and Entian, 2005). Crabtree effect involves a complex regulation at transcriptional level in terms of repression of utilization of alternative carbon sources different from simple sugars, respiration and Krebs cycle and gluconeogenesis while gens related with glycolic enzymes and hexose transporters are induced. This regulation is the one behind the fermentative capacity that presents *S. cerevisiae* and no the most of non-*Saccharomyces* wine yeasts (Flores et al., 2000).

1.2.2.1. Traditional inoculation with *S. cerevisiae* as sole starter

A successful AF depends on the wine yeast ecosystem present in fermenting must and it is highly related to the imposition of a specific *S. cerevisiae* strain (Albergaria and Arneborg, 2016; Constantí et al., 1997). This is how we can ensure the completion of the fermentation. Sometimes the imposition of a particular *S. cerevisiae* strain is not reached due to problems in grape health status or when musts present high concentration of toxic compounds for yeasts (Barata et al., 2012). In these cases, the inoculation of an exogenous yeast is needed to complete the AF. And, since *S. cerevisiae* is the one that naturally evolves into predominant, is the species traditionally used for this purpose.

However, inoculation is a common practice in cellar because regardless the quality of the microbiota, it ensures a predominant yeast which will drive the fermentation since the very beginning (Ribéreau-Gayon et al., 2006). Moreover, to facilitate the manipulation of yeasts in cellar, companies produce yeast biomass as active dry yeast (ADY). ADY production consist on extreme dehydration of cells (below 8% of water) which are maintained as a state of suspended metabolism (Dupont et al., 2014). After, when ADY is inoculated in wine cells are hydrated and start fermenting. The use of ADY is a common practice not only in winemaking but also in other food industries because it simplifies the manipulation of the microorganisms. Besides, yeasts preserved as ADY are genetically more stable allowing longer periods of storage.

Altogether, inoculation of *S. cerevisiae* is the usual trend to ensure the completion of AF. This brings us to the concepts of spontaneous and inoculated fermentations. Inoculated fermentations ensure wine production and provides a homogeneous quality, whereas spontaneous fermentation is unpredictable (Beltran et al., 2002; Ribéreau-Gayon et al., 2006). In spite of the unpredictable compound of the spontaneous fermentation, the succession of the autochthonous yeasts growing through the changing ecosystem allows more complex sensory attributes. This is mainly because inoculating *S. cerevisiae* in the initial must in a massive population rapidly displace the autochthonous non-*Saccharomyces*. Since non-*Saccharomyces* can present different metabolic fingerprint than *S. cerevisiae*, that particular character of the grape microbiota is lost (Medina et al., 2013; Padilla et al., 2016a).

1.2.3. Non-*Saccharomyces* as starter cultures

In the last decades science has started to pay attention to non-*Saccharomyces* yeasts. Before, the usual trend was to inoculate *S. cerevisiae* as soon as possible to displace the non-*Saccharomyces* growth. Nevertheless, during a spontaneous AF they reach high populations that should significantly impact wine composition somehow apart from *S. cerevisiae* fingerprint itself. At the beginning, this yeast group was related with negative aspects because they were linked to stuck and sluggish fermentations.

Table 1. Positive oenological effects of the most relevant non-*Saccharomyces* genera. The effects are presented with *S. cerevisiae* as reference yeast (adapted from Capozzi et al., 2015).

Yeast genera	Oenological features	References
Candida/ Starmerella	• Reduction of ethanol concentration • Low volatile acidity • High production of glycerol, esters, aldehydes, ketones, terpenes and C13-norisoprenoids • Decreases ethyl acetate, volatile FAs and malic acid compounds • Decreased concentrations of aldehydes and acetate esters • Production of volatile compounds with antifungal activity against *B. cinerea*	Englezos et al. (2015, 2016b, 2016a), Lemos Junior et al. (2016), Nisiotou et al. (2018)
Hanseniaspora	• Increased levels of acetate esters, ethyl esters, MCFA ethyl esters, terpenes, and FA • High production of 2-phenylethyl acetate, glycerol, acetaldehyde and vitisin B • Low volatile acidity • Synthesis of benzoid compounds • Reduced levels of ochratoxin A	Hu et al. (2018a, 2018b), Lombardi et al. (2018), Martin et al. (2016b, 2016a), Medina et al. (2013, 2016), Tristezza et al. (2016)
Issatchekia	• Reduction of malic acid and acetaldehyde • Higher production of phenols, monoterpenes and norisoprenoids	González-Pombo et al. (2011), Kim et al. (2008), de Ovalle et al. (2018)
Kluyveromyces/ Lachancea	• Reduction of the pH and acetaldehyde and fusel alcohols content • Increased lactic acid, glycerol and 2-phenylethanol concentration • Low volatile acidity • Release of Kwkt killer toxins against *Brettanomyces/ Dekkera*	Balikci et al. (2016), Benito et al. (2015), Comitini et al. (2004), Comitini and Ciani (2011), Gobbi et al. (2013), Kapsopoulou et al. (2005, 2007)
Metschnikowia	• Reduction of ethanol and acetaldehyde concentration • High production of esters and glycerol • Increased levels of 4-methyl-4-sulfanylpentan-2-one, 2-phenylethyl alcohol and 2-phenyl acetate • Low volatile acidity • Antimicrobial activity	Contreras et al. (2014) Escribano et al. (2018), Oro et al. (2014), Rodríguez et al. (2010), Ruiz et al. (2018), Sadoudi et al. (2012), Varela et al. (2016, 2017)

Pichia	• Increase of the thiol 3-mercaptohexyl acetate and acetaldehyde • Enhancement of vinylphenolic pyranoanthocianins • Release of killer toxins with antifugal activity • High content of polysaccharides	Anfang et al. (2009) Benito et al. (2011) Blaszczyk et al. (2015), Comitini et al. (2011), Domizio et al. (2011)
Schizosaccharomyces	• Consumption of malic acid • Decrease of the urea content • High production of polysaccharides • Increase pyruvic acid and vinylphenolic pyranoanthocyanin content	Loira et al. (2018), Morata et al. (2012), Mylona et al. (2016), Romani et al. (2018)
Torulaspora	• Reduction of ethanol concentration • Low volatile acidity and acetaldehyde • Increased production of 2-phenylethanol, lactones and glycerol • Decreased FA and ethyl ester concentration • Consumption of L-malic acid • Low production of fusel alcohols • High mannoprotein production • Release of TdKT killer toxins against spoilage yeast	Azzolini et al. (2012, 2015), Belda et al. (2015), García et al. (2017), González-Royo et al. (2015), Villalba et al. (2016)
Zygosaccharomyces	• Low production of acetic acid, H_2S and SO_2 • Degradation of malic acid • High production of polysaccharides	Domizio et al. (2011), Jolly et al. (2014)

New knowledge about non-*Saccharomyces* identification and typification techniques, allowed to start studying their real impact on wine quality (Petruzzi et al., 2017). In last few years studies about their particular enzymatic activities have been carried out (Belda et al., 2017b). Considering *S. cerevisiae* as model wine yeast, non-*Saccharomyces* present different metabolic pathways and metabolic behaviours in wine. For instance, they have much higher expression of ß-glucosidases which releases odorant compounds from those aroma precursors coming from the grape skins. Also, they are characterized by higher expression of proteolytic and pectolytic enzymes which help colloidal stability and polyphenolic extraction respectively. Moreover, they are generally Crabtree negative which result in lower ethanol yields in comparison with *S. cerevisiae*. Altogether, non-*Saccharomyces* present abilities to modulate wine composition which allow the winemaker to produce a distinguished product (Capozzi et al., 2015). This is of particular interest since inoculation trend with *S. cerevisiae* worldwide has homogenization of product which currently demand differentiated products. Some of the most relevant and best characterised effects of these yeasts in wine are summarized in Table 1.

However, the preparation of this group of yeasts to winemaking was, and still is, difficult for many species. Their low tolerance to ethanol and, especially SO_2, make them difficult to work with under cellar conditions. Also, their application as starter cultures is more recent due to many inconveniences in their production as ADY than *S. cerevisiae* which delayed their commercial availability.

To date, strains of *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Lachancea thermotolerance, Pichia kluyveri* and *Saccharomyces pombe* are commercially available as ADY (Jolly et al., 2006; Petruzzi et al., 2017; Roudil et al., 2020). Besides, some starter cultures consist of a mix of two yeast species (Roudil et al., 2020).

The use of non-*Saccharomyces* as starter cultures is proposed in combination with *S. cerevisiae*. The reason is because non-*Saccharomyces* have less tolerance to ethanol and SO_2 which finally compromises the completion of the AF. Two inoculation strategies are proposed: sequential inoculation and coinoculation.

In sequential inoculation the selected non-*Saccharomyces* strains is inoculated in the initial must. After certain time the selected *S. cerevisiae* strain is then inoculated. Usually, *S. cerevisiae* is inoculated 48 hours after the AF is started by the non-*Saccharomyces*. Other time of regimes are also used, as 24h or 72h. Once *S. cerevisiae* is inoculated and reaches populations of 10^8 CFU/mL, the non-*Saccharomyces* population (inoculated and autochthonous) start losing their viability. The coexistence of the two yeast groups will depend in their particular strain's specific interactions. In the majority of the cases, no viable non-*Saccharomyces* will be detected at the final AF stage as it occurs when just *S. cerevisiae* is inoculated.

The other strategy for the use of non-*Saccharomyces* as starter culture is the coinoculation with *S. cerevisiae*. In this regime of inoculation both strains are inoculated at the same time in the initial must. Usually, the modulation due to the presence of non-*Saccharomyces* is less noticeable because *S. cerevisiae* overcomes rapidly the growth. Consequently, the time that the non-*Saccharomyces* is metabolically active is usually less than in sequential inoculation.

In both cases, mixed fermentation with non-*Saccharomyces* and *S. cerevisiae* produces longer AF.

1.2.3.1. *Torulaspora delbrueckii*

T. delbrueckii is one of the first non-*Saccharomyces* available as ADY and is one of the most commercially available yeast species (Table 2). It is a yeast that can be found in late stages of AF due to its high resistance to ethanol and SO_2. It also presents a high metabolic activity under winemaking conditions as *S. cerevisiae*. Indeed, *T. delbrueckii* and *S. cerevisiae* are genetically close (Masneuf-Pomarede et al., 2016).

Table 2. Commercially available strains from *T. delbrueckii* and *M. pulcherrima* (adapted from Roudil et al., 2020).

Product	Providing company	Advantages	References
T. delbrueckii			
Biodiva	Lallemand	• Increases perception of some esters without masking the typicity. • Low volatile acidity	Bagheri et al. (2017), González-Royo et al. (2015), González et al., (2018a), Medina-Trujillo et al. (2017), Wang et al. (2016), Whitener et al. (2015, 2016, 2017)
Oenoferm® wild & pure	Erbslòh	• It brings a creamy texture with a pleasant lasting mouthfeel.	König and Claus (2017)
Oenovin Torulaspora Bio	Oeno	• It increases the olfactory notes of red fruit and it improves the softness and roundness of wines.	-
PRELUDE™	Christian Hansen	• It guarantees flavour complexity by producing medium chain (stable) fatty acid esters and by promoting MLF. • Production of high concentration of mannoproteins that give a fuller and smoother mouth feel.	Benito (2018b, 2018a) König and Claus (2017), Vong and Liu (2017)
PRIMAFLORA®	AEB GROUP	• Protection of the must from spoilage microorganisms by competitive selection. It brings some aromatic complexity, and it improves mouthfeeling.	Loira et al. (2014), Simonin et al. (2018)
Q Tau	Enartis	• Production of high amounts of esters and terpenoids that create fresh, red fruits aromas. Production of low volatile acidity. It increases smoothness.	-
Viniferm NSTD	Agrovin	• Intensification of the perception of floral aromas by producing β-phenyl ethanol. Production of high amounts of mannoproteins.	Belda et al. (2016, 2017a), Tronchoni et al. (2018)
ZYMAFLORE® Alpha	Laffort	• Production of varietal thiols. Low production of volatile acidity.	Chasseriaud et al. (2018), Loira et al. (2014), du Plessis et al. (2017), Renault et al. (2015, 2016), Sun et al. (2014)

M. pulcherrima			
Excellence® BIO-NATURE	Lamothe-Abiet	• Production and control of the indigenous microbiota.	-
Flavia	Lallemand	• Releases of thiols and terpenic compounds during AF, favorising the expression of red and white wines.	Benito (2018b), Benito et al. (2015), Chasseriaud et al. (2018), González-Royo et al. (2015), González et al. (2018a), Whitener et al. (2015, 2016)

As specific wine modulation examples, *T. delbrueckii* can reduce ethanol content, reduce the volatile acidity, decrease fatty acid concentration, increase mannoprotein concentration, increase glycerol concentration and increase the production of fusel alcohols in mixed fermentations together with *S. cerevisiae*. Particularly regarding to wine aroma compounds, *T. delbrueckii* metabolic activity helps to release terpene aromas such as α-terpineol and linalool (Čuš and Jenko, 2013). Besides, the use of *T. delbrueckii* is related to an enhancement in the fruity character of wines (Morata et al., 2020).

It can also be interesting in the production of sparkling wines because it increases foamability and foam persistence (González-Royo et al., 2015).

1.2.3.2. *Metschnikowia pulcherrima*

M, pulcherrima is a common wine yeast worldwide found in the first stages of wine AF. This yeast species exhibits high antimicrobial activity, specially consequence of pulcherrimic acid, the pigment which turns into red its colony when growing on plate (Oro et al., 2014). That is why apart from its use as starter culture in winemaking it is proposed as promising control agent against pathogenic fungi as *Botrytis* or other wine spoilage yeast as *Brettanomyces* (Freimoser et al., 2019; Oro et al., 2014). Nevertheless, this specific character is not observed under winemaking conditions since it rapidly loses viability in favour to other non-*Saccharomyces* and *S. cerevisiae*. Still, some commercial strains are currently available (Table 2).

The main oenological potential of this yeast is the capacity of lowering the ethanol content of wines. It has low ethanol yield since it is Crabtree negative and present a highly active glyceropyruvic pathway (Contreras et al., 2014). Also, *M. pulcherrima* can

increase mannoprotein concentration in wine and foam persistence (González-Royo et al., 2015).

According to wine aroma, it can notably increase aroma compounds due to its ß-glucosidase and ß-xylosidase activities (Fleet, 2003; Hernández-Orte et al., 2008; Padilla et al., 2016b) which particularly increase the concentration of monoterpenols and finally, contribute to enhance the fleshness of wine (Morata et al., 2020).

1.3. Malolactic fermentation and *Oenococcus oeni*

MLF is a biotransformation driven out by LAB in alcoholic fermented products like wine and cider (Lorentzen and Lucas, 2019). This fermentation usually takes place after the AF but can occur also during it. It consists on the decarboxylation of L-malic acid into L-lactic acid (Kunkee, 1991; Lerm et al., 2010; Pilone and Kunkee, 1970). The biotransformation results in the conversion of a dicarboxylic acid into a monocarboxylic acid which results in a reduction of acidity, thus, in an increase in the pH (Liu, 2002; Lonvaud-Funel, 1999; Wibowo et al., 1985).

Even though this process is known as MLF, it is not a fermentation but an enzymatic decarboxylation without other intermediary molecules. LAB conduct this enzymatic decarboxylation through the malolactic enzyme (MleA). For the reaction, MleA requires of two cofactors: (i) Mn^{2+} cation and (ii) NAD^{+}. ME is formed by two protein subunits of 60 kDa (Ansanay et al., 1993). The substrate for this biocatalysis is L-malic acid. However, other structurally analogous organic acid present in wine (succinic acid, citric acid or tartaric acid) can act as competitive inhibitors for the active site of MleA (Ribéreau-Gayon et al., 2006).

As MLF reduces acidity, it is highly recommended in red winemaking and also in high acidity white wines. Still, MLF is not desirable in low acidic white wines or sparkling wines where the acidity plays a principal role in these wines. Besides, LAB consume other nutrients during MLF impoverishing wine and, therefore increasing microbial stability (Liu, 2002). Nevertheless, as result of their metabolism, LAB can synthesis undesirable compounds that can compromise the organoleptic profile of wine, so MLF has to be a controlled process (Waterhouse et al., 2016). Besides, a

controlled MLF process can also lead to the production of some positive aromas in wine (Bartowsky and Henschke, 2004; Maturano and Saguir, 2017).

Ecological dynamics of LAB have been thoroughly studied for years. Generally, low population LAB density is detected in early stages of AF, coming from grape skins (Ribéreau-Gayon et al., 2006). Bacterial communities (LAB and other environmental bacteria) found in grapes is not randomly distributed, it is dependent on grape varietal, geographical situation and orientation (Portillo et al., 2016). The LAB diversity is maintained until the alcoholic content is not very high. When the ethanol concentration starts to increase, the bacterial population begin to decrease. At that point, contrary to the behaviour of other LAB species, *O. oeni* commence to grow actively (Lonvaud-Funel, 1999). Basically, *O. oeni* becomes dominant because of its high tolerance to ethanol, SO_2, low pH, and other stressful conditions (Wibowo et al., 1985). This dominant bacterium can be found in low proportion in grape bacterial community and also be part of the resident microbiota of the cellar (Franquès et al., 2017; González-Arenzana et al., 2012b).

Moreover, it has been reported that a minimum population of 10^6 cells/mL is needed to start the consumption of L-malic acid (Lonvaud-Funel, 1999). Under the stressful environmental conditions (Fleet et al., 1984) that present wine, the failure of MLF is usual. Trying to solve this problem, similarly to what happens in the AF, the starter culture technology was developed with LAB (Antalick et al., 2013; Henick-Kling and Park, 1994; Jussier et al., 2006). For this purpose, *O. oeni* was selected as candidate due to its high adaptation to wine conditions. In addition to the selection of tolerant wine strains, there is an increasing consciousness of the potentially effects of the interaction between yeast strains used to perform AF and the ability of the LAB bacteria to carry out the MLF. That is why the yeast- *O. oeni* strain compatibility begin to be considered as another criteria for wine strain selection.

1.3.1. Wine lactic acid bacteria

Lactic acid bacteria (LAB) are gram positive with low G+C content, *id est*, from the phylum *Firmicutes* (Figure 7). Particularly, LAB are nonsporing, catalase negative, aerotolerant, acid tolerant and strictly fermentative rod or coccus which produce lactic

acid as major end product (Ribéreau-Gayon et al., 2006). Besides, they present high nutritional requirements (Terrade and Mira de Orduña, 2009) which limit their ecosystem to rich media such us vegetables or dairy products.

Their principal metabolism is the fermentation of sugars. In basis of this fermentation, they are divided into homofermentative and heterofermentative. Homofermentative species produce lactic acid as sole end product whereas heterofermentative species produce a mixture of lactic acid, ethanol, acetate and CO_2 (Ribéreau-Gayon et al., 2006).

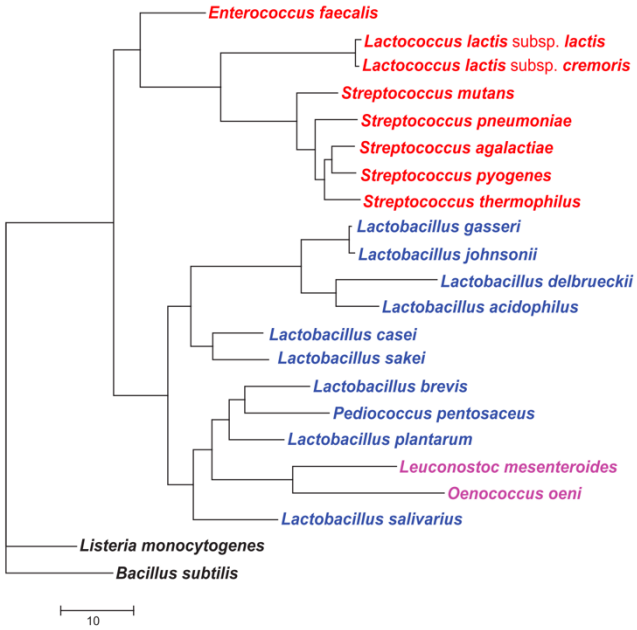

Figure 7. A phylogenetic tree of some LAB constructed on the basis of concatenated alignments of four subunits (α, ß, ß' and δ) of the DNA-dependent RNA polymerase. The maximum-likelihood un- rooted tree was built using the MOLPHY program (1). All branches are supported with >75% bootstrap values. The species are coloured according to Makarova et al. (2007) differentiation in *Lactobacillaceae*, blue; *Leuconostocaceae*, purple; *Streptococcaceae*, red (From Makarova et al., 2007).

Phylogenetically, and according the database of NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), LAB involves around 500 species of the order *Lactobacillales*, belonging to five families: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae* and *Streptococcaceae* (Makarova and Koonin, 2007; Zhang et al., 2011).

The main genera of LAB found in winemaking are *Lactobacillaceae* of genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Ribéreau-Gayon et al., 2006).

From these genera, the main oenological interest species are *La. plantarum* and *O. oeni*.

The population of LAB found in fresh grape must is very low around 10^2-10^3 CFU/mL (Costello et al., 1983; Fleet et al., 1984), mainly *Lactiplantibacillus* and *Oenococcus* (Franquès et al., 2017). This population is usually inhibited during the AF because the high concentration of ethanol and SO_2 related with the high metabolic activity of wine yeasts. After AF, when the yeasts start to die and also autolysate, LAB begin to grow (Ribéreau-Gayon et al., 2006). The biodiversity of the initial must is reduced during AF ethanol increases. As result of this increasing selective pressure, the best wine LAB adapted species: *O. oeni* is found as predominant.

Contrary to the AF, MLF may require long time to be performed. It can last months until the LAB population reaches the threshold of 10^6 CFU/mL to start L-malic acid consumption (Lonvaud-Funel, 1999). Still, populations of more than 10^6-10^7 CFU/mL are needed to undergo MLF in harsh conditions (Lorentzen and Lucas, 2019).

1.3.1.1. *O. oeni*, the main species in malolactic fermentation

O. oeni, formerly called *Leuconostoc oenos* (Dicks et al., 1995) is an heterofermentative wine LAB. It is one of the four species of the genus *Oenococcus* together with *O. kitahareae*, isolated from shochu residues (Endo and Okada, 2006), *O. alcoholitolerans* (Badotti et al., 2014) isolated from cachaça fermentation and bioethanol plants and the recently described *O. sicerae* (Cousin et al., 2019) isolated from cider.

O. oeni presents a little genome (1.9 Mb) in comparison to other wine LAB as *L. plantarum* (3.3 Mb) or even the model LAB, *B. subtilis* (4.1 Mb). This little genome allows to this particular bacterium to mutate and generate high diversity of strains. Up to date, May 2021, there are 244 different genomes of *O. oeni* available in the database of NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). PSU-1 (ATCC BAA-331) strain was the first sequenced genome from this species (Neeley et al., 2005) and currently is used as model strains. Currently, there are also 4 other strains (UBOCC-A-315001, UBOCC-

A-315001, CRBO_1381 and 19) completely sequenced in 2018 and 2019 (Breniaux et al., 2018).

Wine is the most common ecology niche of *O. oeni* (Lorentzen and Lucas, 2019). Considering the physicochemical conditions present in wine, *O. oeni* is highly adapted to acidic and ethanol stresses (Margalef-Català et al., 2016a). Moreover, wine is a limited nutrient source to grow in. After AF, there is barely nothing but L-malic acid that this LAB can use as energy source.

As the most adapted LAB in wine, *O. oeni* is usually used as starter culture to undergo the MLF. Nevertheless, the inoculation of *O. oeni* is not always enough to complete MLF in wines exhibiting harsh conditions.

1.3.1.1.1. *O. oeni* diversity

O. oeni is considered as a fast-evolving bacteria species (Yang and Woese, 1989). Its genome does not code for *mutS* and *mutL* genes which are involved in DNA mismatch repair system (Mills et al., 2005). As result of this, high diversity of *O. oeni* strains has been reported due to possible spontaneous mutations. These can be the reason to its high adaptability to certain niches.

Traditionally, *O. oeni* biodiversity was assessed based on pulsed-field gel electrophoresis of large DNA fragments produced by restriction enzyme digestion of the bacterial chromosome (REA-PFGE) or Rapid Amplification of Polymorphic DNA (RAPD) (Lorentzen et al., 2019). Currently the preferred technique is Multiple Loci VNTR Analysis (MLVA); amplification and length analyses of variable number of tandem repeats (VNTR) of polymorphic genomic regions (Claisse and Lonvaud-Funel, 2012, 2014). All these techniques allowed to unreveal the high diversity present in this species. Of course, all of them are limited to distinguish strains among a group but not to classify or compare with other groups. This problem has been solved with the use of genome sequencing. Since 2005, when the first genome of *O. oeni* was released, more than 200 hundred genomes are available. Besides, recently *O. oeni* strains have been classified in bases on Multi Locus Sequence Typing considering the sequences of constitutive genes to assess diversity. This technique clustered strains in 4 (A-D) genetic lineages (Lorentzen et al., 2019) where two of them (A and B) are the

main ones. Group A involves wine strains, groups B and C cider and wine strains and, finally group D kombucha strains.

During the winemaking process the biodiversity of *O. oeni* is highly modified. Larger number of strains are detected in initial must than in the final wine after MLF. Still, the ecosystem passes through a complex evolution where strains successively appear and disappear. Moreover, not always there is a single predominant strain detected but a sort of 4-6 strains which appears as main strains (Reguant et al., 2005a). As consequence, it is difficult to determine the origin of a particular strain during the whole winemaking process (González-Arenzana et al., 2012b). Studies in this field demonstrated that some strains of the grape must come from grapes and other can have their origin in cellar. Even if *O. oeni* has complex nutrient requirements, it can survive by producing exopolysaccharides and biofilms in cellar surfaces (Bastard et al., 2016; Dimopoulou et al., 2014). These is particularly interesting because some commercial strains, which are selected because of their well-adapted phenotype, can survive from vintage to vintage in the cellar and colonize musts the following years.

1.3.2. Malolactic fermentation by *O. oeni*

As previously presented, LAB population need to reach at least a population of 10^6 CFU/mL to start L-malic acid consumption. In most cases, the LAB identity will be completely *O. oeni* as only fermenting species.

In *O. oeni* malic acid is decarboxylated in the cytosol (Ribéreau-Gayon et al., 2006). The substrate enters the cell through a permease (MleP) as monoanionic malate ($HMal^-$) versus a carrier-independent efflux of lactic acid (HLac) (Figure 8). Once the malate in the cytosol ($HMal^-$), it is decarboxylated where a proton (H^+) is consumed in the catalysis and HLac is produced and released. As consequence, the intracellular pH increases and also an electrochemical gradient ($\Delta\psi$) is generated due to the accumulation of $HMal^-$.

The produced $\Delta\psi$ together with the ΔpH result in the creation of a proton motive force (PMF) which enables the entrance of H^+ through ATPase for the synthesis of ATP. The entrance of 3 H^+ generates sufficient energy to produce from ADP and inorganic phosphate a molecule of ATP.

This metabolism is organized in an operon where the gen of the malolactic enzyme (*mleA*) and the malate permease (*mleP*) are found (Figure 8). These genes are preceded by *mleR* which encodes for a transcriptional regulatory protein of the operon.

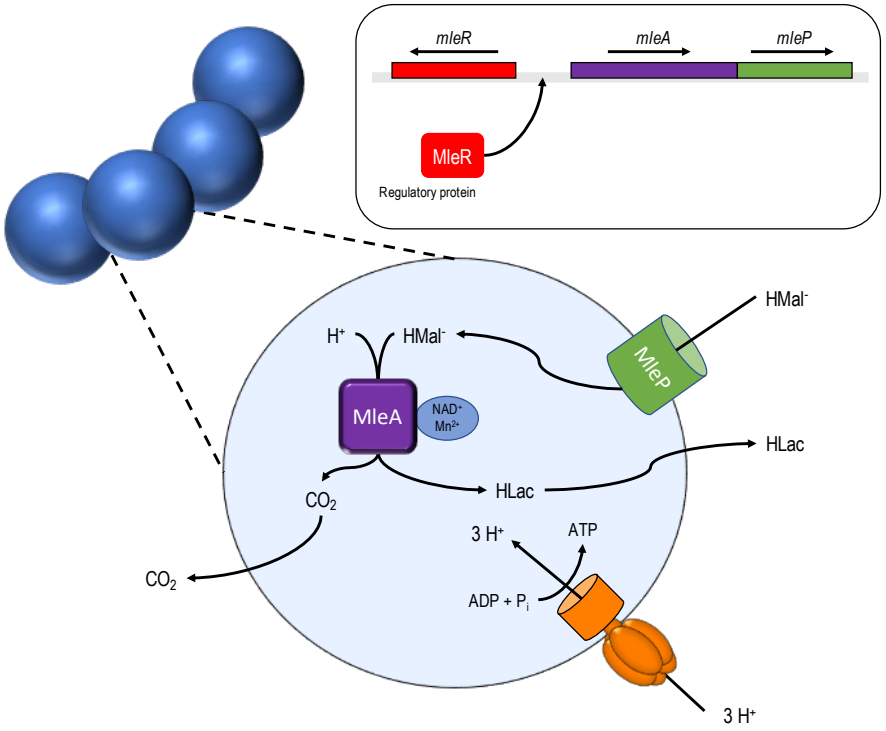

Figure 8. Graphical representation of the malolactic fermentation process undergone by an *O. oeni* cell. Inside the box is represented the operon in which all proteins involved in the process are codified.

Under these extreme conditions where MLF takes place the failure of MLF is usual. The metabolism *O. oeni* exhibits and its stress response are not always sufficient. That is why inoculation is a possible solution to ensure MLF performance. Inoculated strains are selected in terms of highly developed stress responses, quick MLF and positive metabolism attributes to enhance organoleptic profile (Antalick et al., 2013). Since not all wines are equal, there exists the need to select a particular *O. oeni* strain for each wine.

1.3.3. Survival of *O. oeni* in wine

Wine is a highly stressful medium to propagate. Low pH, ethanol and limit nutrient availability are the common denominator of the oenological media. Besides, other compounds intrinsically related with wine; polyphenols, can contribute to difficult *O. oeni* growth and metabolism in red winemaking. This heterogenic group are generally related with difficult MLFs (Reguant et al., 2000) and they are recently described as

stress compounds (Bech-Terkilsen et al., 2020). Their effect depends on their structure (Devi and Anu-Appaiah, 2018; García-Ruiz et al., 2011) and the particular sensibility of the *O. oeni* strain (Zimdars et al., 2021)

Under these harsh environment, *O. oeni* has adopted it as main ecological niche. It has developed adaptation mechanisms to resist the harsh conditions of wine. The most evident mechanism is MLF but there are others which are related to the metabolism adaptation, to produce energy, and stress responses to overcome the difficulties to grow in wine.

1.3.3.1. Metabolism

Wine has very limited energy sources. Still, *O. oeni* and other LAB are capable of growing in the absence of sugars even if they present a much more profitable fermentation behaviour in other media (Ribéreau-Gayon et al., 2006). One of the most abundant energy sources is ethanol which can be oxidized by acetic acid bacteria but not by LAB. L-tartaric acid is the next most abundant constituent of wine which other LAB but *O. oeni* can use. Then, it comes to L-malic acid which is the preferred energy source for *O. oeni*, the substrate of MLF.

Citric acid apart from been considered as a competitor for the active site of MleA, it can also be catabolized by *O. oeni* and other LAB (Bartowsky and Henschke, 2004). This metabolism is only observed as response to acidity or ethanol stress (Olguín et al., 2009). CitP and MaeP antiporters exchange dianionic citrate ($HCit^{2-}$) from the extracellular with monoanionic lactate (Lac^{-}). The $\Delta\psi$ generated is added to the one obtained with MleP and contributes to produce PMF. Once in the cytosol citrate is divided into oxaloacetate (and acetyl-CoA), decarboxylated to pyruvate, transformed to α-acetolactate and fermented to acetoin and 2,3-butanediol as end products. This end products can directly impact in wine organoleptic profile by enhancing the butter aromas. However, high concentration of them are reported as undesirable (Bartowsky and Henschke, 2004; Davis et al., 1985). In this way, under semi-aerobic conditions α-acetolactate be chemically oxidized to diacetyl with has a lower aroma threshold. Still, under anaerobic conditions, typical state of wine fermentation, α-acetolactate is transformed to acetoin and then reduced to 2,3-butanediol (Waterhouse et al., 2016).

Traces of sugars found in wine after AF can also be metabolized by *O.oeni*. These monosaccharides as glucose or fructose, which can represent less than 2 g/L together, can be uptake by the bacterium and be active substrates for the phosphotransferase system (PTS) (Jamal et al., 2013). Even if low sugar concentration is available in wine, *O. oeni* possess mannosidase enzymatic activity that allow mannose release from mannoproteins (Jamal et al., 2013). Mannoproteins are the main polysaccharide from the yeast cell wall released to wine during AF (Vejarano, 2020), and especially during ageing (Belda et al., 2016). Studies in yeast derived compounds have demonstrated a stimulatory effect on *O. oeni* growth in presence of these macromolecules (Diez et al., 2010; Guilloux-Benatier et al., 1995; Liu et al., 2017b). Nowadays, we can relate this positive effect due to an uptake of mannose hydrolysed from mannoproteins, which can be substrate of the PTS system (Cibrario et al., 2016; Jamal et al., 2013). The main function of PTS is to translocate sugars or sugar alcohols across a membrane with simultaneous phosphorylation but without the implication of concentration gradient (Jamal et al., 2013).

Apart from carbon, nitrogen sources are also necessary for the bacterium biosynthetic pathways to develop. Nitrogen composition in wine includes proteins, peptides, and free amino acids. Protein concentration is low and usually remains without modifications because they are not hydrolyzed by wine microorganisms. After AF, the concentration of free amino acids, as it occurs with simple carbon sources is very low. It can represent less than 20 mg/L of nitrogen (Gobert et al., 2017; Roca-Mesa et al., 2020). The largest source of nitrogen in wine are peptides. Peptides can represent up to 100 mg N/L in finished wine and they are going to be the preferent nitrogen source for *O. oeni* in wine. The bacterium is able to break down those peptides and release free amino acids into the wine (Remize et al., 2006). Indeed, it can grow with peptides as sole nitrogen source. Due to this particularity, wines after MLF can present higher amino acid concentration than quantified after AF (Alcaide-Hidalgo et al., 2008).

In addition, *O. oeni* is able to metabolise amino acids. For instance, it can catabolise arginine through arginine deiminase (ADI) pathway (Liu et al., 1995). The metabolization of arginine can lead to the eventual production of precursor compounds of ethyl carbamate and putrescine (Araque et al., 2011). Ethyl carbamate

is a carcinogenic compound related with amino acid metabolism of yeasts and LAB in fermented beverages (Ubeda et al., 2020).

After MLF, the concentration of esters usually increases. This is due to the esterase activity that *O. oeni* and other LAB possess (Cappello et al., 2017) and greatly depend on the media (Fia et al., 2018). This esterase activity is more active in producing short-chained esters (C2-C8) related to SCFA, MCFA, fusel alcohols and organic acids (Cappello et al., 2017; Davis et al., 1988). Besides, ethyl acetate, isoamyl acetate, and ethyl lactate are the most produced esters during MLF (Antalick et al., 2012; Costello et al., 2013; Maturano and Saguir, 2017; Ugliano and Moio, 2005).

1.3.3.2. Stress responses

The survival and successful adaptations are linked to some modifications that the cell has to commit. These changes are the result of a genetic regulation to that particular environment which appear suddenly in the medium. Generally, are the response to toxic compounds or lack of nutrients from growth in optimal conditions. Consequently, the stress respond will fit to the particular stress it has to be faced. The molecular mechanisms of stress response have been studied by means of transcriptional studies or using omic approaches that revealed genes up or down regulated or proteins, which are more or less abundant (Margalef-Català et al., 2016a; Olguín et al., 2015). Besides, as one of the most important cell structures to preserve the integrity of the bacterium in wine like conditions is cell membrane, studies focused in membrane anisotropy are also common (Maitre et al., 2014; Margalef-Català et al., 2016b). There are also other promising studies using antisense RNA technology (Darsonval et al., 2015).

The particular response of *O. oeni* in wine is mainly related with acidic and ethanol response to guarantee the integrity of the cell under winemaking conditions.

1.3.3.2.1. Cell membrane and wall

Ethanol interacts with the lipids present in the cell membrane affecting its fluidity and processes. The main functional categories affecting are metabolite transport and cell wall and membrane biosynthesis (Margalef-Català et al., 2016a; Olguín et al., 2010). Due to the presence of high concentrations of ethanol in wine, the membrane

of *O. oeni* tends to be more fluid and, consequently affecting some processes. The perturbed membrane has less control of its functions and leads to a leakage of intracellular components, as cofactors and ions and also affects the formed electrochemical gradient. So, *O. oeni* has to rigid the membrane.

Cell membrane, which is mainly a bilayer of phospholipids, has also a vast number of different fatty acids (FA). The FA concentration and type: saturated (SFA), unsaturated (UFA) and cyclopropane fatty acids (CFA); regulates the fluidity of the membrane. In presence of 10% of ethanol, *O. oeni* reduces the UFA/SFA and increases the protein/lipid ratios (Garbay et al., 1995; Garbay and Lonvaud-Funel, 1996), resulting in the formation of a more rigid membrane. Besides, the biosynthesis of CFA is promoted, specifically lactobacilli acid by activating the gene *cfa* which transforms UFA in CFA (Grandvalet et al., 2008; Teixeira et al., 2002).

Some small heat shock protein (sHsp) appear to be essential to the maintenance of cell membrane fluidity. Particularly, Lo18 in ethanol and heat stress has been shown to interact with cell membrane and liposome fluidity (Guzzo et al., 1997).

Cell membrane is surrounded by the cell wall which helps to maintain cell integrity and form. The activation of some enzymes linked to the biosynthesis of some constituent of the wall (N-acetylmuramoyl-L-alanine amidase, ADP glucose 6-deshydrogénase) (Ribéreau-Gayon et al., 2006) under ethanol stress revealed the role of the wall in ethanol stress response (Margalef-Català et al., 2016a).

1.3.3.2.2 ATPase system

Bacteria cells depends on the ATP synthase or ATPase for producing energy. This complex is a membrane-bound enzymatically active protein complex which allows ion transport. The transport of protons through the channel can be in favour to the gradient with production of energy (ATP) or contrary to the gradient with consumption of energy (Lerm et al., 2010)

The ATPase activity as way of producing energy has been introduced before as the consequence of the PMF derived from the MLF metabolism. Besides, ATPase is in charge of maintaining the intracellular pH around 6. It is the main mechanism on the acid tolerance of the bacteria. When growing in acid media, where in wine it is a pH

lower than 4, it is crucial to extrude protons out of the cell to keep the cytosolic processes at pH 6. In the opposite way of producing ATP, the hydrolysis of an ATP allows 3 protons to exit the cell through the ATPase (Ribéreau-Gayon et al., 2006).

For this purpose, *O. oeni* has a membrane type (F_0F_1) H^+-ATPase: a F_1 cytoplasmic complex (subunits α, β, δ, Ɛ and γ) which contains the catalytic site for ATP hydrolysis and a F_0 integral membrane complex (subunits A, B and C) which forms a proton channel. This activity is highly affected by the active mechanism of SO_2 used in winemaking and produced during AF by yeasts (Carreté et al., 2002).

1.3.3.2.3. Redox systems

O. oeni an anaerobic aerotolerant LAB. Even if it does not use oxygen in its metabolism, it is exposed to little concentrations of oxygen during winemaking. Still, the oxygen concentration is low considering the fermentative capacity of yeasts which produce high amounts of CO_2 that displace the dissolved oxygen. is not highly exposed to oxygen during winemaking.

Oxygen is a highly reactive molecule which can be partially reduced to reactive species of oxygen (ROS): superoxide anions ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radicals (OH^-) or hydrogen peroxide (H_2O_2). Cumulative ROS is known as oxidative stress and can cause high damage in the cell. These ROS can attack proteins, lipids and nucleic acid causing cell ageing or, eventually cell dead (Su et al., 2015).

Mechanisms that detoxify ROS are oxidoreduction systems (or redox). LAB inactivate ROS through enzymatic activities: NADH oxidase/peroxidase system, superoxide dismutase and catalase (Bruno-Bárcena et al., 2004; Kullisaar et al., 2010; Su et al., 2015). Besides, thioredoxins (Trx) or low molecular-weight thiols including glutathione (GSH), can participate in these redox systems. To date, the redox systems mainly studied in *O. oeni* are Trx and GSH systems (Holmgren, 1985; Margalef-Català et al., 2016b, 2017b; Su et al., 2015).

1.3.3.2.4. Stress proteins

The biological response of cells involves a complex regulation of gene expression. In this sense the production of small proteins is a quick response under the pressure

of some stress while larger proteins are in synthesis. These group of proteins are named heat shock proteins (HSP) because they limit the denaturalization and aggregation of intracellular proteins in difficult environmental conditions. Also, their nomenclature includes a number which represents its weight in KDa. They are classified in 3 families of HSP: (i) proteins with chaperon activity, (ii) Clp proteins or caseinolytic proteins and (iii) small HSP which form oligomers with chaperone activity that prevents polypeptides from aggregation (Carreté et al., 2005).

These stress proteins are located in specific genomic regions, which are regulated by the master regulator CstR (Grandvalet et al., 2005). This master regulator responds to wine like conditions and is responsible of the expression of *groESL* and *dnaK* operons, *clpP*, *clpC*, *clpL2*, *clpX* and *hsp18* genes (codifying for Lo18 protein). These genes present different activation patterns. In this sense, *clpX* is preferentially expressed at the beginning of the exponential phase (Jobin et al., 1999), whereas *hsp18* at the end of the same phase (Grandvalet et al., 2005). Indeed, the most known sHSP in *O. oeni* is Lo18 protein, which is activated after heat (42 ºC), acidic (pH 3) and ethanol (12% vol/vol) shocks (Guzzo et al., 1997; Jobin et al., 1997).

Moreover, Clp proteins, which are ATPase dependent proteases, are involved in *O. oeni* survival at high temperatures. They are divided in two groups: (i) those with two ATP nucleotide-binding-domains (NBD), as ClpA, ClpB, ClpC, ClpD, ClpE, and ClpL, and (ii) those with just one NBD, like ClpX (Schirmer et al., 1996).

There are also *groESL* and *DnaK* operons that have been related with the production of stress proteins, encoded in *O. oeni*, found in PSU-1 strain (Mills et al., 2005). Increased transcriptional levels were observed for *groES* and *grpE* under heat (42°C) and ethanol shock (11% v/v) also under acidic shock (pH 3.6) for *grpE* (Desroche et al., 2005; Grandvalet et al., 2005). Besides, similar to what happens with Lo18 (Weidmann et al., 2010), DnaK seems to be recluit in the membrane as result of ethanol shock since its cytosolic concentration decreases (Olguín et al., 2015).

1.3.3.2.5. Exopolysaccharides

O. oeni genome encodes different genes related to the synthesis of exopolysaccharides (EPS). EPS are extracellular polymers composed of sugar monomers which vary depending on the strain and the growth medium.

The adaptation of *O. oeni* to low content of ethanol induce a production of an EPS layer. It creates a layer which protects the cell from desiccation, osmotic acid or cold stress and against toxic compounds such as ethanol or SO_2 (Dimopoulou et al., 2014).

1.4. Possible yeast- *O. oeni* interactions in wine: role of non-*Saccharomyces*

The influence of yeasts upon *O. oeni* is determinant since *O. oeni* grows in the medium produced by yeasts during AF. In general, most of the compounds produced by yeasts during AF will negatively impact upon *O. oeni*. Those compounds, such as ethanol, SO_2, low pH and MCFA, will develop a stress response in *O. oeni*. Besides, their concentration will depend on the AF strategy used. In general terms, the resulting wine will be consequence of *S. cerevisiae*'s metabolism since it is the one yeast usually inoculated. So, any changes in the inoculated yeast will generate different wines changing the effect towards *O. oeni*.

As the research in non-*Saccharomyces* increase, the nature of these interactions is more clarified. Unfortunately, few studies cover the whole picture of yeast-*O. oeni* interactions, since not much of them study in the same experiment the impact of the changes of the yeasts upon a MLF undergone in the same wine. The most common scenario is to integrate two studies: (i) wine modulation due to the use of a particular yeast and (ii) the impact will have in *O. oeni* according to the general knowledge of its metabolism. The main disadvantage of this pipeline is that the medium has a great impact on the nature of these interactions. And what is more, it is almost impossible to assign the effect of a particular compound in such a complex matrix as wine.

Nevertheless, authors agree that the type and impact of the interactions is dependent on several factors like (i) the initial must composition, (ii) the yeast/bacteria strain combination, (iii) the uptake and release of nutrients by yeasts, and (iv) the ability of yeasts to produce metabolites that affect somehow LAB (Alexandre et al.,

2004; Balmaseda et al., 2018; King and Beelman, 1986; Lonvaud-Funel et al., 1988; Du Plessis et al., 2017). Still, the effect of some compounds is not yet clear due basically to the lack of information towards the impact in *O. oeni* metabolism.

Some authors suggest the coinoculation of yeasts and *O. oeni* in the initial must to mitigate the harsh conditions of wine after AF (Sumby et al., 2014). Nevertheless, it is not a common practice and the improvement on the MLF is limited or unclear (Izquierdo-Cañas et al., 2014).

1.4.1. Chemical mediators of the interactions

As previously stated, wine is complex environment full of inhibitor compounds. Besides, the lack of nutrients makes the media harsher for *O. oeni*. So, the particular consumption of some nutrients during AF will determine the nutrient availability for the MLF. *O. oeni* has extremely complex nutritional requirements (Terrade and Mira de Orduña, 2009) and the availability of such nutrients, for instance amino acids, will depend on the media (Arnink and Henick-Kling, 2005; Tristezza et al., 2016) and the preferences of the inoculated yeasts (Ivey et al., 2013). High fermentation performance yeasts present high amino acid consumption (Roca-Mesa et al., 2020). As consequence, wines produced with *T. delbrueckii,* or *S. cerevisiae* will have low amino acid content.

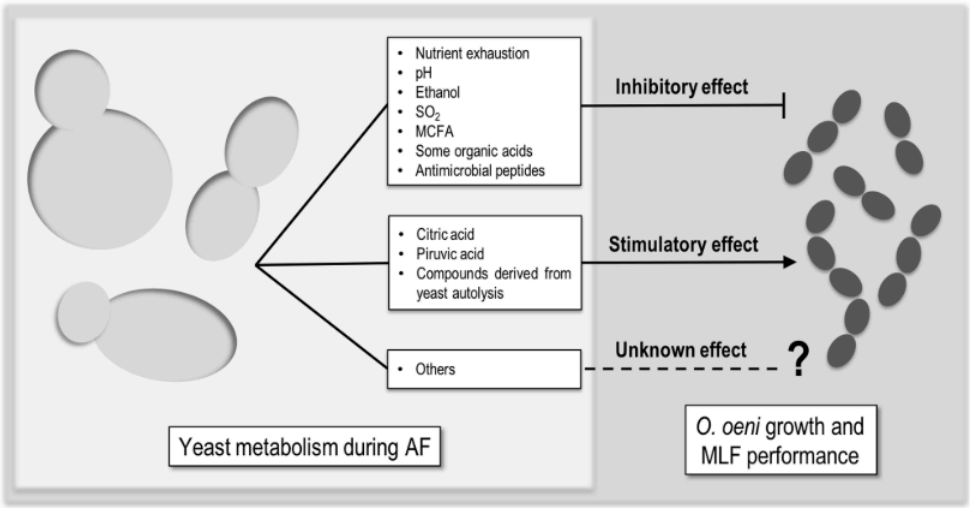

Figure 9. Wine compounds and parameters related with yeast metabolism with known effect upon *O. oeni* and MLF performance (from Balmaseda et al., 2018)

On the contrary, *M. pulcherrima* or *H. uvarum* require less quantity of amino acids, which would be beneficial for *O. oeni*. Nevertheless, the competition between two yeast

species can lead to higher consumption of nutrients in *S. cerevisiae* impoverishing much more the media, if possible (Curiel et al., 2017). In this sense, *O. oeni* does not need large quantities of amino acids or peptides as nitrogen source to perform MLF, thus, nitrogen concentration is usually not a problem (Remize et al., 2005).

Wine chemical compounds related with yeasts' metabolism can be classified in terms of the effect upon *O. oeni* and MLF (Balmaseda et al., 2018). This classification (Figure 9) can evolve and be complemented as the role of yeast metabolites interactions with *O. oeni* is unrevealed or clarified.

1.4.1.1. Inhibitor compounds

Ethanol as main inhibitor compound of yeast in wine present high importance in the compatibility with *O. oeni*. Also, it plays a negative role in costumers health. For this reason, the reduction of ethanol is a current trend. One of the most promising strategies to achieve that goal is the use of non-*Saccharomyces* with low fermentative performance (Padilla et al., 2016b). Among this vast group of yeasts, some authors suggest the use of *T. delbrueckii* (Benito, 2018a), *Starmerella bacillaris* (Englezos et al., 2016a) or *M. pulcherrima* (Contreras et al., 2014) among others.

Sulphur dioxide is another chemical compound widely found in wine with antimicrobial activity. This compound, which causes a decrease in the ATPase activity in *O. oeni* (Carreté et al., 2002), can be reduced by the use of non-*Saccharomyces*. Generally, these yeasts have lower tolerance to SO_2 (Jolly et al., 2014) and, therefore, they are related with a lower production of it in regard to *S. cerevisiae*. It has to be considered that the amount of SO_2 produced as consequence of yeast metabolism, is usually lower that the limit concentration *O. oeni* can tolerate and nothing compared to usual oenological additions. In this sense, some authors have related *H. uvarum* and *T. delbrueckii* with higher SO_2 concentrations (Belda et al., 2015; Ferrando et al., 2020). In contrast, Martín-García et al., (2020) reported a slight reduction in free SO_2 in mixed fermentations with *M. pulcherrima* and *T. delbrueckii*. Altogether, the concentration of SO_2 will be also very dependent of the nitrogen composition (Osborne and Edwards, 2006).

Studies of the inhibitory effect of MCFA in *O. oeni* were early reported (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988). These molecules can alter the fluidity of the membrane and difficult MLF performance. Generally, non-*Saccharomyces* can reduce the content of MCFAs in wine. These reductions are particularly due to hexanoic and octanoic acids (Fairbairn et al., 2021). Strains bellowing to the species *H. uvarum* (Hu et al., 2018a, 2019; Liu et al., 2016), *I. orientalis* (Liu et al., 2016), *L. thermotolerans* (Fairbairn et al., 2021) or *M. pulcherrima* (Hranilovic et al., 2020) in mixed fermentation are reported to reduce MCFAs in regard to *S. cerevisiae* control vinification. In contrasts one of those studies also reported higher concentrations with *M. pulcherrima, C. stellata*, and *P. fermentans* (Liu et al., 2016). Besides, as happens with sulphur dioxide, MCFA production is highly affected by the nitrogen composition of musts (Hu et al., 2019).

pH is one important oenological parameter modulated by the use of non-*Saccharomyces*. Some species as *T. delbrueckii* and *M. pulcherrima* are related with increasing its value (Martín-García et al., 2020), which was dependent of the inoculation regime. Others as *C. stellata, L. thermotolerans, S. pombe* or *S. bacillaris* are actually used to increase the acidity of wines (Berbegal et al., 2019) as a solution to manage the effects of climate change (Ubeda et al., 2020). Other authors present no differences when comparing with *S. cerevisiae* wine (Ferrando et al., 2020)

Succinic acid is usually related with MLF inhibitory effect through a competitive inhibition by the active site of the malolactic enzyme (Davis et al., 1985; Lonvaud-Funel and Strasser de Saad, 1982). Recently, some studies in non-*Saccharomyces* have been published with different reports. Hranilovic et al., (2020) showed a high increase in succinic acid in *M. pulcherrima* wines, whereas Martín-García et al., (2020) observed a slight reduction in mixed fermentations with the same yeast and *T. delbrueckii*. Also, Ferrando et al., (2020) did not observe changes in regard to *S. cerevisiae*. Another study using different non-*Saccharomyces* reported increased concentrations in *H. uvarum* (Harlé et al., 2020).

The use of non-*Saccharomyces* is related with higher concentrations of polyphenolic compounds due to their particular enzymatic activities, which can increase the extraction of grape skins, and due to lower adsorption by yeast cell walls.

Particularly, *T. delbrueckii* has shown an increase on anthocyanin concentration in red wines (Benito, 2018a; Escribano-Viana et al., 2019) even if this behaviour seems to be strain specific (Carew et al., 2013; Chen et al., 2018). Careful attention must be paid to this increase in polyphenolic compounds since they can be inhibitors for *O. oeni* (Bech-Terkilsen et al., 2020; Reguant et al., 2000).

Up to date, there is no literature available concerning the production of antimicrobial peptides in non-*Saccharomyces* with potential effect in *O. oeni*. It is easy to suppose that there should be some as found in *S. cerevisiae* (Branco et al., 2014, 2017; Díez et al., 2012) with effect on the membrane of the bacterium. Besides, some non-*Saccharomyces* are recently related with the production of this kind of compounds (Vejarano, 2020) but little is known about them and their mechanisms.

1.4.1.2. Stimulating compounds

Stimulating compounds are usually related with energy sources that *O. oeni* can use under oenological conditions apart from L-malic acid. Glucose and fructose could be related with this effect but their increase or presence in wine due to non-*Saccharomyces* makes no sense in winemaking. Besides, other sugars and carbon sources can be present.

One of them is citric acid. The consumption of this organic acid is related with a stress metabolism in *O. oeni* (Olguín et al., 2010). No many studies report citric acid concentration modulation due to non-*Saccharomyces*. The few of them report similar concentrations in *M. pulcherrima* and *T. delbrueckii* mixed fermentations respect to *S. cerevisiae* wines (Belda et al., 2017b; Martín-García et al., 2020; Ruiz et al., 2018). Also, Ferrando et al., (2020) observed higher concentrations in *S. bacillaris* wines. Nevertheless, there is no report that relates higher citric acid concentrations with better MLF performances.

Another organic acid that can play a stimulatory role in *O. oeni* is pyruvic acid. This is another intermediary of yeast metabolism that can be used as external electron acceptor, facilitation the regeneration of NAD^+ (Maicas et al., 2002) or promote diacetyl production (Mink et al., 2015). Several species are related with the increase of pyruvic acid. For instance, *S. bacillaris* (Ferrando et al., 2020), *L. thermotolerans*

(Benito et al., 2019; Del Fresno et al., 2017), *T. delbrueckii* (Benito et al., 2016), *S. pombe* (Benito et al., 2019) or *H. uvarum, M. pulcherrima* and *M. fructicola* (Harlé et al., 2020).

From the vast group of molecules that can be released as result of the autolytic process of yeasts, mannoproteins are of especial interest. As stated before, these macromolecules can be hydrolysed and the released mannose can be uptake by *O. oeni* and used as substrate of the PTS system (Jamal et al., 2013). Several authors have recently reported higher concentrations of mannoproteins when using non-*Saccharomyces*. Not only in ageing (Belda et al., 2016), but also after AF (Ferrando et al., 2020; Vejarano, 2020). Belda et al., (2016) observed a dramatic increase in mannoprotein content when using *T. delbrueckii*, *M. pulcherrima* and an overproducer *S. cerevisiae* strain compared to a reference *S. cerevisiae* strain, similar to what Ferrando et al., (2020) observed with these two species. Since these macromolecules are related with a stimulatory effect upon *O. oeni* (Diez et al., 2010; Guilloux-Benatier et al., 1995) that increase due to non-*Saccharomyces* should have an enhanced positive phenomenon in MLF (Balmaseda et al., 2018).

1.4.2. Wine organoleptic attribute modulation

Apart from the positive or negative effect of non-*Saccharomyces* on *O. oeni*, there is also another concern which is important to consider. The microbial interactions of oenological yeasts and LAB in wine are decisive in the organoleptic profile of the product. As result, the yeast and LAB tandem compatibility is a key point of the fermentative process.

Few works deeply study this concern attending to some relevant compounds directly related with the organoleptic profile of wines, as those related with wine colour and aroma. Moreover, the wine tasting is crucial to verify if those changes significantly impact in consumers appreciation. Nevertheless, it can also happen to have a discrimination by the wine tasting and not be able to identify it with a specific compound (Nardi et al., 2019). Moreover, the selection of the inoculation regimes is determinant in the chemical modulation using non-*Saccharomyces* and LAB. Russo et al. (2020) observed that the use of *S. bacillaris* coinoculated with *O. oeni* and *S.*

cerevisiae in must had a positive effect in some relevant wine attributes, while sequential inoculation was not that convenient.

Colour is one of the main concerns in red winemaking. The change in pH after MLF dramatically impacts on the colour compositions as it alters the equilibrium of the pigments (Glories, 1984). The use of non-*Saccharomyces* together with selected LAB can modulate this wine attribute (Nardi et al., 2019). Thus, the use of selected non-*Saccharomyces* able to increase the polyphenolic compounds (Escribano-Viana et al., 2019), in order to mitigate the loss of colour due to the pH increase after MLF (Costello et al., 2012), can be an interesting strategy for red winemaking.

To sum up, the AF inoculation strategy not only modulates the organoleptic profile of wines, but also impacts on the development of MLF. We have seen that some non-*Saccharomyces* showed a promising compatibility by both, stimulating MLF and producing wines different from *S. cerevisiae* fermented ones. Thus, the knowledge on the nature and mediators of these interactions could led to a practical application in the management of MLF.

2. Hypothesis and objectives

The main target of study has been the impact of non-*Saccharomyces* in wine parameters and its effects and interactions with *Oenococcus oeni* and malolactic fermentation (MLF). As the use of non-*Saccharomyces*, specially *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, as starter cultures for the alcoholic fermentation (AF) increases, the understanding of impact in *O. oeni* is crucial to ensure the MLF.

Wine is a complex matrix in which *O. oeni* has to propagate and survive in order to complete MLF. The harsh conditions found in wine extremely difficult the process especially due to low pH, high ethanol concentration and polyphenols (Bech-Terkilsen et al., 2020). The yeasts that have conducted AF greatly impact wine chemical composition, and thus, they can affect to the development of MLF. This thesis aim was to expand the knowledge of the interactions between non-*Saccharomyces* and *O. oeni* in terms of chemical mediator modulation and *O. oeni* adaptation mechanisms related with them to assess and better understand their effects in MLF performance.

Under this new oenological context, the **hypothesis** of this thesis was that using non-*Saccharomyces* yeast in AF has some relevant effects on *O. oeni* and that some of these effects are positive for the efficient development of MLF and for the final quality of wine. Thus, the main objective of the thesis was to clarify the role of non-*Saccharomyces* in the yeasts-*O. oeni* interactions and how they impact in wine characteristics and how *O. oeni* responds to them. To assess this main goal, the following specific objectives were attained to:

Objective 1: assess the consequences of interactions between non-*Saccharomyces* yeasts and *O. oeni* in wine quality and MLF performance in cellar winemaking (Chapter I: 1, 2)

Objective 2: characterize the influence of *T. delbrueckii* in *O. oeni* biodiversity (Chapter I: 1, 2; Chapter II)

Objective 3: clarify the effect of yeast derived compounds, especially mannoproteins, in *O. oeni* growth and MLF performance related with the use of non-*Saccharomyces* (Chapter III: 1, 2)

Objective 4: identify the most relevant molecular mechanisms of *O. oeni* involved in wine adaptation influenced by non-*Saccharomyces* (Chapter IV)

Objective 5: address the main changes in nitrogen metabolism of *O. oeni* affected by *T. delbrueckii* (Chapter V)

This thesis contributes to the general knowledge of the main consequences of yeasts-*O. oeni* interactions in winemaking. This knowledge will help to better understand the impact of non-*Saccharomyces* chemical modulation in MLF performance. The possible application of this thesis could help to define the most important criteria in the selection of yeast-*O. oeni* tandem starter cultures for a successful inoculation strategy in wine industry.

3. Results

CHAPTER I

Oenological impact of interactions between non-*Saccharomyces* and *Oenococcus oeni* under cellar conditions

In this chapter, we studied the oenological consequences of interactions between non-*Saccharomyces* and *O. oeni* in some relevant wine attributes and fermentative process under cellar conditions. This chapter is divided in two consecutive vintage experiments (vintage 2018 and 2019) through two manuscripts.

In vintage 2018 (**Chapter I: 1**) we performed sequential inoculations with *T. delbrueckii* and *M. pulcherrima* in white (Macabeo) and red (Cabernet Sauvignon) grape musts, and subsequently inoculated *O. oeni* to undergo the MLF.. We observed a decrease in MLF duration using non-*Saccharomyces* and a global organoleptic modulation, especially in volatile compounds. Besides, we detected an increased polyphenolic composition, mainly due to anthocyanins, in non-*Saccharomyces* fermented wines. This particular aspect made us to deeper study this phenomenon.

Thus, in vintage 2019 (**Chapter I: 2**) we focused on these interactions related with polyphenolic composition in red winemaking. We performed sequential inoculations with just *T. delbrueckii*, described as more suitable for red wines, in grapes with two maturity levels (optimal and before optimal). In this experiment, we observed a reduction in MLF duration in *T. delbrueckii* fermented wines and similar organoleptic modulation by the use of different inoculation strategies as observed in vintage 2018. Interestingly, *T. delbrueckii* enabled spontaneous MLF in high polyphenolic wines, which did not occur in *S. cerevisiae* wines. These findings confirmed *T. delbrueckii* as an interesting fermenting yeast for enhance polyphenolic composition and promote MLF in difficult wines.

CHAPTER I: 1

Impact of changes in wine composition produced by non-*Saccharomyces* on malolactic fermentation

Aitor Balmaseda[a], Nicolas Rozès[b], Miguel Ángel Leal[a], Albert Bordons[a], Cristina Reguant[a,*]

* Corresponding author: cristina.reguant@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

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Abstract

Non-Saccharomyces yeasts have increasingly been used in vinification recently. This is particularly true of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, which are inoculated before *S. cerevisiae*, to complete a sequential alcoholic fermentation. This paper aims to study the effects of these two non-*Saccharomyces* yeasts on malolactic fermentation (MLF) carried out by two strains of *Oenococcus oeni*, under cellar conditions. Oenological parameters, and volatile and phenolic compounds were analysed in wines. The wines were tasted, and the microorganisms identified. In general, non-*Saccharomyces* created more MLF friendly conditions, largely because of lower concentrations of SO_2 and medium chain fatty acids. The most favourable results were observed in wines inoculated with *T. delbrueckii*, that seemed to promote the development of *O. oeni* and improve MLF performance.

Keywords

Non-*Saccharomyces*, malolactic fermentation, *Oenococcus oeni*, wine

Introduction

Wine is the result of the alcoholic fermentation (AF) of grape must in a complex microbial environment. In the first stages of AF, a high diversity of yeast genera are involved: *Hanseniaspora, Pichia, Torulaspora, Metschnikowia* and *Starmerella,* among others (Beltran et al., 2002; Capozzi et al., 2015). When the ethanol concentration begins to increase, in the presence of added SO_2, a process of yeast selection begins, with *Saccharomyces cerevisiae* being the final predominant species. In this scenario, fermentation performance is highly influenced by the species fermenting the must (Capozzi et al., 2015; Comitini et al., 2011; Morata et al., 2019). To better control the process, it has long been proposed that *S. cerevisiae* be used as the most preferred yeast starter culture in must (Fleet and Heard, 1993).

During or after AF, malolactic fermentation (MLF) with lactic acid bacteria (LAB) as drivers can occur. This fermentation consists of the decarboxylation of L-malic acid to L-lactic acid. The species that dominates the process is *Oenococcus oeni* (Davis et al., 1985). MLF improves the quality of wine since this biotransformation increases pH, enhances organoleptic properties and has a positive role in microbial stabilisation (Lerm et al., 2010; Lonvaud-Funel, 1999). So, MLF is usually desirable in red wines or highly acidic white wines.

Since inoculation has become a usual cellar practice, *S. cerevisiae* has been used as the most preferred commercial starter culture. Research into non-*Saccharomyces* yeasts has increased in the last decade, and some of them have been proposed as starter cultures (Roudil et al., 2020). These non-*Saccharomyces* usually cannot finish AF, so they are inoculated with *S. cerevisiae*. The usual strategy is first to inoculate the non-*Saccharomyces* yeast and then *S. cerevisiae* (González-Royo et al., 2015; Jolly et al., 2014). In this sequential inoculation, the time that the non-*Saccharomyces* ferments by itself will determine the wine characteristics (Martín-García et al., 2020). Another good strategy is to co-inoculate both yeasts into the initial must (Azzolini et al., 2012; Belda et al., 2015; Ciani et al., 2016; Comitini et al., 2011; Jolly et al., 2014; Renault et al., 2015).

Among non-*Saccharomyces* that have been used most are *T. delbrueckii*, *M. pulcherrima* or *Lachancea thermotolerans,* which are available to oenological

companies as starter cultures (Roudil et al., 2020). There are also available commercial strains of *Lachancea thermotolerans*, *P. kluyveri* and *Schizosaccharomyces pombe* (Jolly et al., 2006; Petruzzi et al., 2017). Other used non-*Saccharomyces* have been *St. bacillaris* (synonym: *Candida zemplinina*) and *H. uvarum* (anamorph *Kloeckera apiculata*) (Ciani and Maccarelli, 1998; Comitini et al., 2011; Englezos et al., 2019; Giaramida et al., 2013; Kapsopoulou et al., 2005; Du Plessis et al., 2017). Most of these species modulate the chemistry of wine by releasing aroma (Belda et al., 2017; Ramírez et al., 2016), which, among other things, decreases the ethanol concentration (Belda et al., 2017b; Contreras et al., 2014), and increases glycerol and mannoprotein concentrations (Belda et al., 2016; Benito et al., 2015; González-Royo et al., 2015).

The chemical characteristics of wine are the consequence of the metabolism of the yeasts that dominate the AF. So, yeasts can positively, neutrally or negatively affect MLF performance (Balmaseda et al., 2018). It is difficult to classify the interactions between yeasts and *O. oeni* since these effects are highly influenced by the media -the grape matrix or the synthetic must or wine- and the strains (Alexandre et al., 2004). The studies that have been carried out showed that non-*Saccharomyces* chemical modulation affects *O. oeni* and MLF performance (Alexandre et al., 2004; Englezos et al., 2019; Martín-García et al., 2020; Du Plessis et al., 2017; Ramírez et al., 2016). Of all the non-*Saccharomyces* species described as modulators of the organoleptic profile of wine, *T. delbrueckii* and *M. pulcherrima* are related with positive chemical changes in the development of MLF (Balmaseda et al., 2018; Ramírez et al., 2016). These changes are always referred to AF carried out with *S. cerevisiae* as sole starter. This is why these two species are of particular interest for stimulating the performance of MLF in harsh oenological conditions. Notably, in sequential inoculation with *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* can produce wines with less ethanol content (Belda et al., 2016; Contreras et al., 2014; Morata et al., 2019), and higher mannoprotein concentration (González-Royo et al., 2015), and can decrease both the acetic acid and SO_2 concentration and increase the pH (Martín-García et al., 2020).

However, there is little information about how these interactions can affect wine quality after MLF under cellar conditions. For this reason, the aim of the present paper was to study the effects of non-*Saccharomyces* yeasts and *O. oeni* interactions in red and white winemaking, with particular focus on *T. delbrueckii* and *M. pulcherrima*.

Material and Methods

Microorganisms and inocula

Three commercial yeast strains (Lallemand Inc., Montréal, Canada) were used: *T. delbrueckii* Biodiva (Td), *M. pulcherrima* Flavia (Mp) and *S. cerevisiae* Lalvin-QA23 (Sc). Yeasts were stored at 4ºC as active dry yeasts provided by the manufacturer. For *O. oeni*, PSU-1 (ATCC BAA-331) and Viniflora CH11 (Chr. Hansen Holding.AS, Hoersholm, Denmark) were used. *O. oeni* was maintained on MRSmf plates (Margalef-Català et al., 2017a) and stored at 4°C. To obtain the inocula, a colony was picked from the plates and grown in liquid media at 27°C in a 10% CO_2 atmosphere (*O. oeni*). Then, 500 µL was inoculated in 50 mL of the same fresh liquid media.

Fermentation trials

Fermentations were carried out with white Macabeo and red Cabernet Sauvignon grape varieties (*Vitis vinifera* L.) during vintage 2018 from the AOC Tarragona (Spain) in the experimental cellar of the Rovira i Virgili University. About 100 kg of each grape variety were manually harvested. Macabeo grapes were destemmed and crushed and finally pressed using the cellar machinery and the resulting must was sulphited with 2.5 g/hL $K_2S_2O_5$ (Fisher Scientific, Madrid, Spain), transferred to a new container and cooled for 24h at 4ºC. Then, the clarified juice was transferred to the fermentation tanks (10 L). Cabernet Sauvignon grapes were destemmed and berries were randomly distributed in 9 batches of 6.5 kg. Each batch was crushed and distributed in food-grade plastic containers, used as fermenters. Then, the crushed grapes were sulphited (2.5 g/hL $K_2S_2O_5$). Fermenting white and red musts were supplemented with nutrients (0.2 g/L Nutrient Vit Nature™, Lallemand Inc., Montréal, Canada) when half the sugars were consumed. Red wines were punched down every 48 hours during the alcoholic fermentation using a stirring tool, moreover the grape skins were always submerged thanks to a flat strainer used as stopper in the fermenter.

Alcoholic fermentations were carried out with two non-*Saccharomyces* strains and by inoculating *S. cerevisiae* after 48h. In accordance with the manufacturer's instructions, each yeast was inoculated for a population of $2.5 \cdot 10^6$ cells/mL with active

dry yeast. There was also a control fermentation with *S. cerevisiae* as the sole starter (Sc). All fermentations were performed in triplicate. Samples were taken every 48h to monitor the decrease in density and the evolution of the yeast population. YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac Química SLU, Castellar del Vallès, Spain) was used to calculate the total number of yeast cells present, and lysine agar medium (Oxoid LTD., Basingstoke, UK) was used to quantify the non-*Saccharomyces* yeasts (Wang et al., 2016), after incubation at 28°C for 48h. AF was considered to have finished when the sugar concentration was below 2 g/L. Macabeo fermentations were carried out at 18 ºC and Cabernet Sauvignon fermentations at 22 ºC.

After AF, the wines were transferred to a new container, cooled for 5 days and decanted. Then, samples were taken centrifuged and stored at -20ºC. In the case of Cabernet Sauvignon, the wines obtained were first pressed. Later, equal volumes of each triplicate (0.5 L) were blended, and 1.5 L was bottled and sulphited (1 g $K_2S_2O_5$/hL) in two 0.75 L bottles. These bottles were stored at 4 ºC until tasting. The residual volume of the mixed wines was supplemented with L-malic acid to achieve a concentration of 2 g/L. Then, the pH was corrected to the value before L-malic acid addition. Adjusted wines were inoculated with two *O. oeni* strains, each in 1 L flasks at 20 ºC to have a population of 10^7 cells/mL. There was also an uninoculated MLF (spontaneous). These fermentations were also carried out in triplicate. Samples were taken every 24h to monitor the consumption of L-malic acid and the evolution of the bacterial population. Samples were plated on MRS_{mf} supplemented with 100 mg/L nystatin (Panreac Química SLU, Castellar del Vallès, Spain), 25 mg/L sodium azide (G BioSciences, St. Louis MO, USA) and 100 mL/L of tomato juice (Aliada, Madrid, Spain) and incubated at 27 °C in a 10% CO_2 atmosphere for 7-15 days. MLF was considered to have finished when the L-malic acid was below 0.05 g/L. After MLF, samples of each triplicate were centrifuged and stored at -20 ºC. Also, 1.5 L of the mixture of the triplicates was bottled, sulphited and stored as described above.

Yeast identification

Twenty-five colonies were randomly selected and isolated from the following samples: must before inoculation, must before inoculating Sc (48h) and wine at the

end of AF (density below 995 g/L and residual sugars below 2 g/L). Isolates were identified, on the basis of the amplicon size of the ITS-5.8S rDNA region, to species level (Esteve-Zarzoso et al., 1999).

LAB identification and strain typing of *Oenococcus oeni*

At least 25 colonies were randomly selected for LAB identification from the following samples: must before inoculation, wine at the end of AF and at the end of MLF (L-malic acid below 0.05 g/L). The LAB were identified and the *O. oeni* typed as described in (Franquès et al., 2018). Briefly, LAB isolates with cocci morphology were confirmed to be *O. oeni* by species-specific PCR according to (Zapparoli et al., 1998). Non-*Oenococcus* isolates were identified with the 16S-ARDRA method and MseI digestion according to (Rodas et al., 2003). The isolates identified as *O. oeni* were typed by the multilocus variable number tandem repeat (VNTR) method based on (Claisse and Lonvaud-Funel, 2014).

For LAB identification by 16S-ARDRA and for *O. oeni* typing, DNA was extracted with the High Pure PCR Template Preparation Kit (Roche, Barcelona, Spain).

Analysis of general oenological parameters

Wines after AF and after MLF were characterised. Concentration of sugars (glucose and fructose), L-malic acid, acetic acid, glycerol, D- and L-lactic acids, primary amino nitrogen (NOPA), NH_4, total and free SO_2, succinic acid and citric acid were determined by enzymatic methods using Miura One Multianalyzer (TDI, Barcelona, Spain). pH was determined using a Crison micro pH 2002 pH-meter (Hach Lange Spain, l'Hospitalet, Spain) and alcoholic content was determined by ebulliometry (Electronic ebulliometer uEBU6576, GabSystem, Moja, Spain) in accordance with the *Compendium of International Methods of Analysis of Musts and Wines* (OIV, 2009).

Analysis of volatile compounds

Wine samples (10 mL) were taken after AF and MLF. The volatile compounds were liquid/liquid extracted with 0.4 mL dichloromethane and 2.5 g $(NH_4)_2SO_4$ using 4-methyl-2-penthanol (0.8 g/L) and heptanoic acid (0.7 g/L) as internal standards,

following Ortega et al., (2001). All reagents were analytical grade from Sigma-Aldrich (St. Louis MO, USA). After 90 min agitation at room temperature and centrifugation (6,000 rpm, 5 min), the organic phase was extracted and 2 µL was injected in split mode (10:1, 30 mL/min) into a gas chromatograph (Agilent Technologies, Germany) with a FFAP column of 30 m × 0.25 mm × 0.25 µm. The temperature of the program started at 35ºC during 5 min, was then increased by 3 ºC/min to 200 ºC, and finally 8 ºC/min to 220ºC. The temperatures of the injector and detector were 180 ºC and 280 ºC, respectively. The gas carrier was He at 3 mL/min. Aromatic volatile compounds were identified and quantified by comparison with standards.

Colour parameters and phenolic determination

Colour parameters (A420, A520 and A620) of wine samples were analysed in a 1 mm cuvette as reported by Glories (1984). CIELab coordinates: lightness (L), chroma (C), hue (h), red-greenness (a) and yellow-blueness (b) were determined in accordance with (Ayala et al., 1997) and data processing was performed with MSCV software (Universidad de la Rioja, Logroño, Spain).

The phenolic composition of red wines was analysed in terms of total polyphenol index (TPI), tannin concentration and anthocyanin concentration. TPI was analysed by measuring the 280 nm absorbance of a 1:100 dilution of red wine with a spectrophotometer. A 10 mm quartz cuvette was used and the absorbance value was multiplied by 100. The tannin concentration was determined using the Bate-Smith method (Ribéreau-Gayon and Stonestreet, 1966) with some modifications (Vignault et al., 2018). The total anthocyanin concentration was determined by the decolouration of wines with sodium metabisulphite (Fisher Scientific, Madrid, Spain).

Wine tasting

Sensory analyses were performed with Macabeo and Cabernet Sauvignon wines after AF and MLF after two months of bottling. Triplicates were blended for simplifying the sensory analysis. Wines were evaluated by 18 trained judges, considered as experts from the Faculty of Oenology of the Rovira i Virgili University. 20 mL of wine were presented in dark glasses to avoid subjectivity by the colour of the samples. Three series of tastings were performed for each type of wine: Sc *vs.* Td, Sc *vs.*

Mp, Td *vs.* Mp. Samples were randomly numbered with 3-digit codes. Wines were served anonymously according to a Latin square of Williams design to avoid range and carry-over effect. Each wine was tasted twice in different series. The descriptive test emphasised the aroma and flavour attributes: lactic character (both white and red wines), fruitiness (both), flowery (white), reduction (both), acidity (both), bitterness (white), astringency (red), balance in mouth (both) and global impression (both). Tasters had to score in a structured scale from 0 (no detection) to 5 (the highest) the intensity of each attribute.

Statistical analyses

The statistical software XLSTAT version 2019.1.2. (Addinsoft, Paris, France) was used. The data obtained was submitted to one-way ANOVA with a subsequent analysis using the Tukey HSD (*Honestly Significant Difference*) test, with a confidence interval of 95% and significant results with a p-value ≤ 0.05. Principal component analyses (PCA) were also performed to determine differences between the wines.

The same XLSTAT software was used to analyse the *O. oeni* genotypic profiles obtained by VNTR with Agglomerative Hierarchical Clustering and Spearman's rank correlation. Genotypes were defined at a minimum similarity level of 95.7% as described by Cruz-Pio et al., (2017).

The results of sensory analysis were submitted to Student's t-test. They were considered significant when the associated p-value was below 0.05. The analyses were performed using PanelCheck software (V1.4.2). PanelCheck software (2006) Nofima Mat, Ås, Norway (http://www.panelcheck.com).

Results and discussion

Fermentation performance

Alcoholic fermentation in Macabeo wines lasted between 21 and 37 days (Table 3). When *S. cerevisiae* was the only yeast inoculated, the fermentation ended first (21 days). The delay in AF was more marked when *T. delbrueckii* was sequentially inoculated (37 days) than *M. pulcherrima* (30 days). These differences were also

reflected in the rate of AF calculated by density drop per day (Table 3). This may be due to the competition between the two starters. Although *T. delbrueckii* is regarded as a good fermenter (Belda et al., 2016), the presence of *S. cerevisiae* in the media can alter its performance (Wang et al., 2016). As a result, the AF takes longer. In the case of *M. pulcherrima* sequential fermentations, the AF may have taken only a little longer because of the higher sensitivity of this particular strain to *S. cerevisiae* (Wang et al., 2016). The prevalence of the non-*Saccharomyces* population, seen by plate counts in lysine agar medium, was the same in Td and Mp wines, and was lost after 19 days. In Sc wine, we did not detect autochthonous non-*Saccharomyces* after 6 days (data not shown).

In the case of Cabernet Sauvignon fermentation, the AF lasted 14 days in all conditions. The fact that the AF lasted less in this red wine than in the white one, and mainly for the non-*Saccharomyces* wines, could be explained by the lower content of nutrients in white wine, and the higher fermentation temperature (22 ºC *vs.* 18 ºC for red and white AF, respectively). It is noticeable that sugar consumption did not start until *S. cerevisiae* had been inoculated in Mp wines (results not shown) after which the AF rate became the quickest (Table 3). As in Macabeo wines, the non-*Saccharomyces* population survived longer (11 days in Td and 10 days in Mp wines) than the autochthonous non-*Saccharomyces* which were viable for 4 days in Sc wines (data not shown).

As far as MLF is concerned, both the inoculated and spontaneous fermentations finished (Figure 10). By the end of AF, in all wines except Sc Macabeo the LAB population was higher than 10^5 CFU/mL (data not shown). In this last wine, the bacterial population was around 10^2 CFU/mL and the spontaneous MLF took 15 days to reach 10^6 CFU/mL and start consuming L-malic acid. At this moment, the acid was consumed very quickly. All MLFs performed in wines previously inoculated with a non-*Saccharomyces* were quicker than the ones performed in Sc wines (Figure 10 and Table 3). This shows that non-*Saccharomyces* somehow diminish the harsh conditions of wine at the end of AF, and in this way these yeasts are beneficial for *O. oeni* and MLF. Mainly in Sc, spontaneous MLF took longer to completely consume the L-malic acid. No great differences were observed in the speed of L-malic acid consumption in red winemaking (Table 3), but statistically it was slower in Sc wines.

Microbial population's analysis

Prior to inoculation, grape musts had a high initial yeast concentration of $1.1 \cdot 10^5$ CFU/mL (YPD) and $5.3 \cdot 10^4$ CFU/mL (Lys) for Macabeo and $3.2 \cdot 10^5$ CFU/mL (YPD) and $1.1 \cdot 10^5$ CFU/mL (Lys) for Cabernet Sauvignon. The implantation of the active dry yeast strains used to inoculate the grape musts was 90% or higher in all cases (data not shown). The inoculations were successful in both white and red wines, and the imposition of non-*Saccharomyces* at 48h (before *S. cerevisiae* was inoculated), and the imposition of *S. cerevisiae* at the end of AF was confirmed. In fact, by the end of AF the non-*Saccharomyces* population had been completely replaced by *S. cerevisiae*.

During MLF, *S. cerevisiae* was still found in populations between 10^3-10^5 CFU/mL (data not shown). After AF, wines were racked, cooled and decanted, but not filtered, so this would explain the presence of viable yeasts. During the time that MLF was carried out, the remaining viable yeasts began to die, losing their ability to grow on YPD plates.

Small concentrations of LAB were detected in must ($1.8 \cdot 10^2$ CFU/mL in Macabeo and less than 10^2 CFU/mL in Cabernet Sauvignon). All wines underwent a successful MLF, including those not inoculated with *O. oeni* (spontaneous MLF). In all cases, the population of the inoculated MLF remained constant at 10^7-10^8 CFU/mL until the end of the fermentation.

The 575 *O. oeni* isolates were typified by VNTRs of 5 polymorphic alleles (Claisse and Lonvaud-Funel, 2014). The VNTR analysis revealed 13 different genotypes (Table 4), two of which – IN1 and IN2 – corresponded to the inoculated *O. oeni* strains PSU-1 and Viniflora-CH11, respectively. Each genotype can be regarded as a different strain. The presence of strain diversity in MLF is a common phenomenon in wine (Lorentzen and Lucas, 2019). In the VNTR profiles obtained, the number of repeats of the alleles varied from 37 to 9 for TR1, 3 to 2 for TR2, 6 to 1 for TR3, 4 to 2 for TR4 and 4 to 1 for TR5.

Table 3. Alcoholic (AF) and malolactic (MLF) fermentation duration and consumption rate. Values shown are the mean of triplicates ± SD. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. PSU-1, CH11 and Spontaneous refer to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated. Statistics were calculated independently for each grape variety.

		Duration (days)				Consumption rate (g/L·day)*			
		AF	PSU-1	CH11	Spontaneous	AF	PSU-1	CH11	Spontaneous
Macabeo	Sc	21	4	8	17	5.67 ± 0.14^{c}	0.55 ± 0.03^{b}	0.25 ± 0.01^{a}	0.61 ± 0.01^{c}
	Td	37	2	2	2	3.06 ± 0.05^{a}	1.03 ± 0.00^{f}	1.03 ± 0.00^{f}	1.03 ± 0.00^{f}
	Mp	30	4	5	5	4.24 ± 0.08^{b}	0.81 ± 0.02^{e}	0.56 ± 0.00^{b}	0.74 ± 0.01^{d}
Cabernet Sauvignon	Sc	14	4	3	5	9.89 ± 0.00^{b}	0.52 ± 0.01^{abc}	0.57 ± 0.01^{cd}	0.50 ± 0.00^{a}
	Td	14	3	3	4	9.28 ± 0.25^{a}	0.66 ± 0.03^{e}	0.66 ± 0.02^{e}	0.56 ± 0.01^{bcd}
	Mp	14	4	3	4	10.72 ± 0.25^{c}	0.61 ± 0.03^{de}	0.65 ± 0.02^{e}	0.51 ± 0.02^{ab}

*Calculation based on consumption rate of sugar as density (AF) and L-malic acid (MLF) considering the period of exponential decrease of these compounds.

$^{a–f}$ Values are significantly different at $p \leq 0.05$. according to a Tukey post-hoc comparison test.

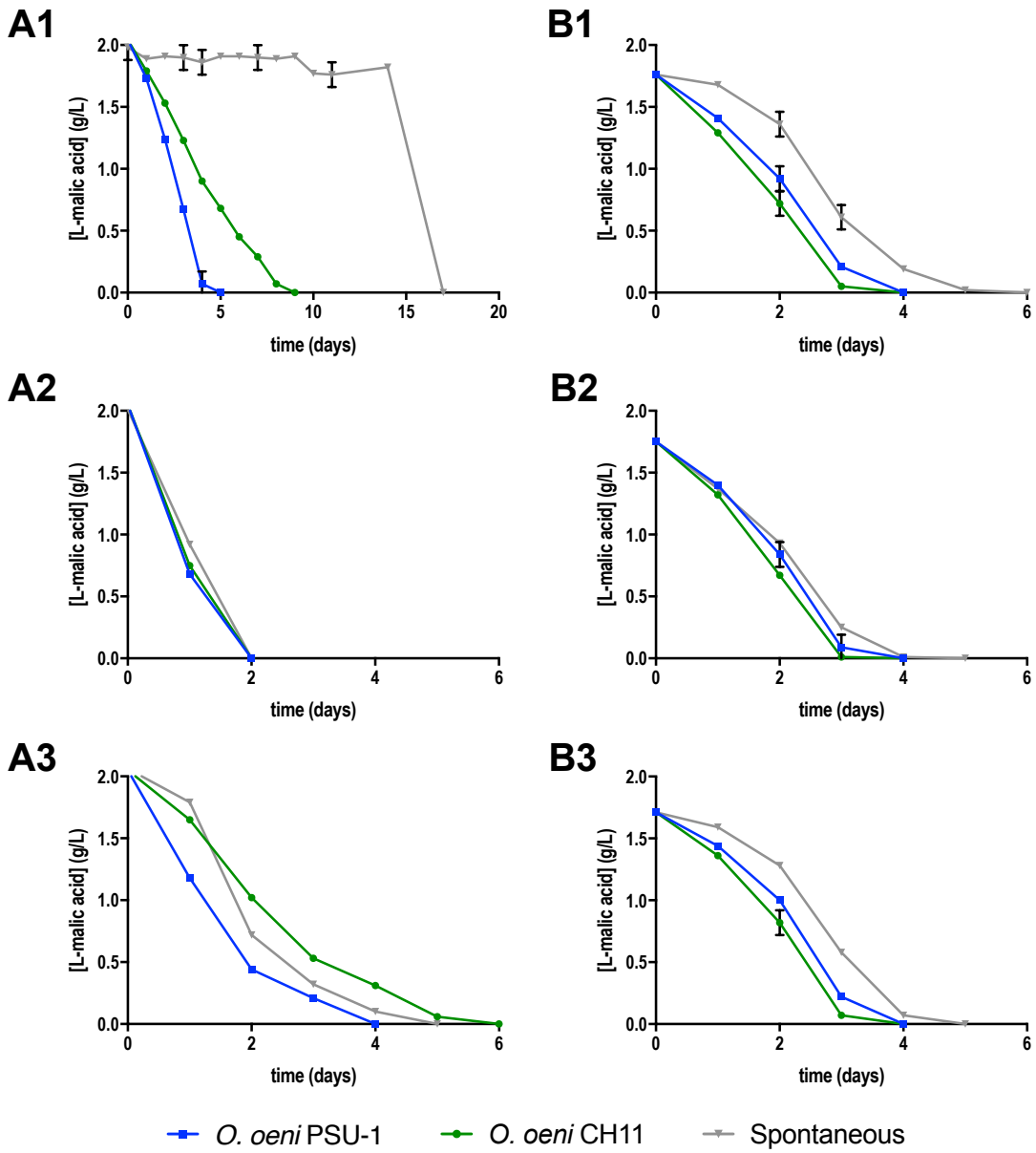

Figure 10. Evolution of malolactic fermentation after AF by monitoring the L-malic acid consumption. Left: Macabeo wines fermented with *S. cerevisiae* (A1), *T. delbrueckii* (A2) and *M. pulcherrima* (A3). Right: Cabernet Sauvignon wines fermented with *S. cerevisiae* (B1), *T. delbrueckii* (B2) and *M. pulcherrima* (B3).

The imposition of the commercial *O. oeni* strains was dependent on the grape variety of the wines (Table 4). Macabeo wines inoculated with *O. oeni* showed, to one extent or another, the presence of each inoculated strain at the end of MLF. Instead, all the Cabernet Sauvignon wines showed the highest imposition percentage for the IN1 genotype (corresponding to the PSU-1 VNTR profile), even in the wines inoculated with the CH11 strain (genotype IN2) and in spontaneous MLF. In fact, the IN1 genotype was already detected in Cabernet Sauvignon at the end of AF before *O.*

oeni inoculation, meaning that the IN1 strain took over the other strains (inoculated or autochthonous) during MLF.

The presence of the IN1 genotype in all Cabernet Sauvignon wines, including spontaneous MLF, indicates that this strain may have adapted to cellar conditions. The type of wine and winemaking practices can modulate the dynamics and prevalence of *O. oeni* strains. As described by several authors, commercial strains previously used in a cellar for several vintages can be detected in wines not inoculated with these strains (El Khoury et al., 2017; Franquès et al., 2017; González-Arenzana et al., 2014; Reguant and Bordons, 2003).

The highest number of different genotypes was detected in Td wines (Table 4). This suggests that the changes in wine composition produced by this yeast would enhance *O. oeni* strain diversity. Altogether, the prevalence of *O. oeni* strains during MLF depends on the type of winemaking (white or red) but also on the yeast species used.

General oenological parameters of wines

The composition of the two musts Macabeo (M) and Cabernet-Sauvignon (CS) was the same in sugars (152 g/L glucose and fructose), acetic acid (0.06 g/L) and NH_4 (30 mg/L), and similar in L- malic acid (1.5 g/L in M, 1.4 in CS) and citric acid (0.33 g/L in M, 0.5 in CS). The main differences were observed in pH, which was lower in Macabeo must (3.42) than in CS (3.75), and primary amino nitrogen (NOPA), which was higher in CS must (50.92 mg/L) than in M must (35.2). It should be noted that the YAN (Yeast Assimilable Nitrogen) for the two grape musts was lower than the accepted limit concentration (140 mg N/L) for finishing AF. Therefore, nitrogen was added in the middle of AF.

The composition of wines showed some differences when different yeast species were used. The main compounds analysed in both Macabeo and Cabernet Sauvignon wines are shown in Table 5. Other compounds, such as sugars, D-lactic acid, nitrogen compounds and succinic acid, were also quantified (Suppl. Table S1), but there were no relevant differences in their concentrations.

Table 4. Percentages of imposition of the different genotypes of *O. oeni* found in wines after AF (grey shaded) and MLF fermentations. IN1 genotype clusters the VNTR profiles similar to *O. oeni* PSU-1. IN2 similar to *O. oeni* CH11. AB named genotypes correspond to naturally appeared (non-inoculated) VNTR profiles clusters. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

	Macabeo									Cabernet Sauvignon										
%	IN1	IN2	AB1	AB2	AB3	AB4	AB6	AB8	AB9	IN1	IN2	AB1	AB2	AB3	AB4	AB5	AB6	AB7	AB10	AB11
Sc			60	40						50		17			33					
Sc-P	29		57	14						67			20			13				
Sc-C	67	17			17					92						8				
Sc-S			93				7			73		18	9							
Td			86	14						50			50							
Td-P	20		73	7						56		6	19	6					6	6
Td-C		60	27	7				7		33		10	19	19	5	5	5	5		
Td-S		6	56	6			33			57		14	21					7		
Mp			62	38						60		40								
Mp-P	67	8	8				17			71		7				21				
Mp-C	7	33	47			7			7	100										
Mp-S		8	92							56			6	13		19	6			

Macabeo wines

The production of acetic acid at the end of AF in Td wines was significantly lower than in Sc and Mp wines. The concentrations were statistically invariable after MLF in all but Mp wines, in which the concentrations increased. During MLF, LAB can metabolise citric acid, which increases the volatile acidity (Franquès et al., 2017; González-Arenzana et al., 2014; El Khoury et al., 2017; Reguant and Bordons, 2003). At the end of AF, citric acid was similar in Sc and Mp wines whereas in Td wines, curiously, the concentration was almost undetectable. During MLF, it was consumed both in Sc and Mp wines by *O. oeni*. However, the increase in acetic acid was more noticeable in Mp wines. Although statistically significant, the increases detected in acetic acid concentration were low and the maximum increase was 0.09 g/L in Mp-P wine. Td wines showed the lowest concentrations of acetic acid because there was no citric acid at the end of AF that could be metabolised by *O. oeni* during MLF.

No differences were found in ethanol and glycerol analyses. In this study we observed no decrease in alcohol content associated with non-*Saccharomyces,* as has been described by other authors (Contreras et al., 2014; Quirós et al., 2014). The behaviour of Td Biodiva was similar to that found in a previous study in which this strain did not decrease ethanol content (Martín-García et al., 2020).

However, in the same study Mp Flavia did significantly decrease it. The ability to reduce ethanol may be dependent on the must and winemaking conditions. The use of non-*Saccharomyces* tended to decrease the pH after AF, and this decrease was significant in Mp wine. After MLF, pH increased as expected due to the decarboxylation of L-malic acid although the pH in Td wines increased less. The lower pH in final Td wines may be because some organic acid compounds were not included in the analysis performed.

Total SO_2 decreased in Td wines after AF as determined in a previous study using the same Td strain (Martín-García et al., 2020). Anyway, the content of total SO_2, always less than 10 mg/L, was much lower than 35 mg/L, the limit of toleration for some of *O. oeni* strains, such as CH11 (Lerm et al., 2010).

Table 5. Oenological parameters of wines after alcoholic (grey shaded) and malolactic fermentations. Values shown are the means of triplicates ± SD. Statistics were calculated independently for each grape variety. Sc, Td and Mp correspond to *S. cerevisiae, T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refer to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

		Acetic acid (g/L)	Citric acid (g/L)	Glycerol (g/L)	SO_2 T (mg/L)	Ethanol (% vol/vol)	pH
Macabeo	Sc	0.4 ± 0.02^{b}	0.15 ± 0.02^{c}	5.12 ± 0.26^{b}	7.00 ± 0.82^{b}	10.5 ± 0.2^{a}	3.17 ± 0.02^{fg}
	Sc-P	0.39 ± 0.01^{b}	0.02 ± 0.02^{ab}	5.27 ± 0.18^{ab}	-	-	3.50 ± 0.03^{bc}
	Sc-C	0.43 ± 0.02^{bc}	0.01 ± 0.01^{ab}	5.28 ± 0.23^{ab}	-	-	3.50 ± 0.00^{bc}
	Sc-S	0.37 ± 0.02^{b}	0.05 ± 0.03^{b}	5.69 ± 0.17^{ab}	-	-	3.26 ± 0.02^{e}
	Td	0.28 ± 0.01^{a}	0.02 ± 0.01^{ab}	5.09 ± 0.08^{b}	2.67 ± 0.47^{a}	10.7 ± 0.3^{a}	3.13 ± 0.02^{g}
	Td-P	0.29 ± 0.01^{a}	0.01 ± 0.00^{ab}	5.25 ± 0.11^{ab}	-	-	3.33 ± 0.01^{d}
	Td-C	0.29 ± 0.02^{a}	n.d.^{a}	5.52 ± 0.39^{ab}	-	-	3.34 ± 0.01^{d}
	Td-S	0.29 ± 0.01^{a}	n.d.^{a}	5.16 ± 0.22^{ab}	-	-	3.24 ± 0.00^{ef}
	Mp	0.38 ± 0.01^{b}	0.16 ± 0.00^{c}	5.60 ± 0.19^{ab}	4.66 ± 0.47^{ab}	10.7 ± 0.2^{a}	2.99 ± 0.03^{h}
	Mp-P	0.47 ± 0.05^{c}	0.02 ± 0.00^{ab}	5.75 ± 0.14^{ab}	-	-	3.63 ± 0.02^{a}
	Mp-C	0.46 ± 0.01^{c}	0.03 ± 0.00^{ab}	5.91 ± 0.15^{a}	-	-	3.61 ± 0.01^{a}
	Mp-S	0.46 ± 0.01^{c}	0.02 ± 0.00^{ab}	5.51 ± 0.20^{ab}	-	-	3.43 ± 0.02^{c}
Cabernet Sauvignon	Sc	0.21 ± 0.07^{a}	0.26 ± 0.11^{b}	6.8 ± 0.53^{a}	9.33 ± 0.58^{b}	10.5 ± 0.2^{b}	3.54 ± 0.02^{b}
	Sc-P	0.42 ± 0.01^{cd}	0.04 ± 0.03^{a}	8.67 ± 0.23^{a}	-	-	4.07 ± 0.02^{d}
	Sc-C	0.45 ± 0.01^{cdef}	0.03 ± 0.02^{a}	7.6 ± 0.2^{a}	-	-	4.08 ± 0.02^{de}
	Sc-S	0.42 ± 0.01^{c}	0.01 ± 0.01^{a}	7.2 ± 0.53^{a}	-	-	4.16 ± 0.01^{ef}
	Td	0.23 ± 0.02^{a}	0.23 ± 0.07^{b}	6.93 ± 1.14^{a}	3.00 ± 1.00^{a}	10.2 ± 0.1^{b}	3.48 ± 0.02^{ab}
	Td-P	0.43 ± 0.03^{cd}	n.d.^{a}	7.27 ± 0.46^{a}	-	-	4.19 ± 0.01^{f}
	Td-C	0.44 ± 0.02^{cde}	n.d.^{a}	7.73 ± 0.12^{a}	-	-	4.23 ± 0.06^{f}
	Td-S	0.32 ± 0.00^{b}	0.02 ± 0.01^{a}	7.8 ± 0.35^{a}	-	-	3.90 ± 0.01^{c}
	Mp	0.28 ± 0.04^{ab}	0.24 ± 0.06^{b}	7.87 ± 0.46^{a}	6.33 ± 3.79^{ab}	10.1 ± 0.1^{a}	3.41 ± 0.08^{a}
	Mp-P	0.51 ± 0.01^{ef}	n.d.^{a}	7.4 ± 1.04^{a}	-	-	4.05 ± 0.00^{d}
	Mp-C	0.50 ± 0.02^{def}	n.d.^{a}	7.2 ± 0.53^{a}	-	-	4.06 ± 0.00^{d}
	Mp-S	0.46 ± 0.01^{f}	0.01 ± 0.01^{a}	7.73 ± 1.45^{a}	-	-	4.18 ± 0.01^{f}

$^{a–f}$ Values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison test.

Cabernet Sauvignon wines

Citric acid was present in similar concentrations at the end of AF and was consumed in all cases during MLF. Consequently, the acetic acid concentration increased to similar amounts in all wines due to *O. oeni* metabolism. The decarboxylation of L-malic acid increased pH, which reached a value close to 4. The high pH of these wines could have enhanced the strain diversity observed. In fact, Td-C wine had the highest pH (4.23) and showed the highest number of different strains at the end of MLF. Total SO_2 at the end of AF was significantly lower in Td wines, and anyway less than 10 mg/L, as for Macabeo wines. This could also have contributed to the greater diversity of *O. oeni* strains observed in the MLF of these wines. In Cabernet Sauvignon vinification, *M. pulcherrima* reduced the ethanol content by 0.5% (vol/vol). This result confirms that the ability of non-*Saccharomyces* to reduce ethanol depends on the type of must and winemaking conditions, since the behaviour of Mp was different in Macabeo wines. The reduction of ethanol could be due to the presence of higher levels of nitrogen and temperature during the fermentation process in Cabernet Sauvignon, compared to Macabeo must.

Volatile compounds

The volatile composition of wines showed that non-*Saccharomyces* had a considerable influence on the organoleptic profile of wines after AF. Both Macabeo and Cabernet Sauvignon wines presented clearly different profiles (Figure 11A and 11C). Interestingly, Td wines were characterised by higher concentrations of 1-butanol, ethyl butanoate, diethyl succinate and 2-methylpropanoic acid in white and red vinifications (Suppl. Figure S1, Suppl. Table S2). Ethyl esters, such as ethyl butanoate and diethyl succinate are compounds considered to be of primary importance for the fruity aroma of wine. Related compounds with aromas have been previously found also in Td wines by other authors (Azzolini et al., 2012; Ramírez et al., 2016; Renault et al., 2015). Fusel alcohol acetates were the only volatiles related to Sc in both wines and 2-methylpropanoic acid in Mp (Table 6, Suppl. Table S2).

The use of non-*Saccharomyces* yeasts reduced the concentration of medium chain fatty acids (MCFA). This reduction was significant in all Td and Mp wines after AF with respect to Sc wines although the differences were more relevant in Macabeo than

in Cabernet Sauvignon wines (Table 6). The release of MCFA by wine yeast can inhibit *O. oeni* growth and malolactic activity and is considered to cause yeast-bacteria antagonism (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988). Capucho and San Romao (1994) reported the inhibitory effect on MLF of decanoic and dodecanoic acids at concentrations above 12.5 and 2.5 mg/L, respectively. The negative impact of MCFA can act synergistically with either low pH and ethanol, inhibiting ATPase activity which is associated to MLF (Carreté et al., 2002). The lower concentrations of MCFA observed in Td and Mp wines in comparison to Sc wines could be due to the action of yeast ghosts generated towards the end of AF of sequential fermentations. In the second half of AF non-*Saccharomyces* viable populations dramatically decreased and dead cells may have adsorbed toxic compounds such as MCFA. In fact, the capacity of yeast ghosts of removing the inhibitory effect of some MCFA has been suggested by several authors (Edwards and Beelman, 1987; Lafon-Lafourcade et al., 1984). The decrease of MCFA was more noticeable in Td Macabeo wines where hexanoic and decanoic acids completely disappeared after AF and octanoic acid was reduced to more than 50% with respect to Sc wines (Suppl. Table S2). These evidences let us to hypothesise that the lowest MCFA concentrations in Td Macabeo wines would have been one of the reasons of a faster MLF than in the rest of the wines.

Ethyl lactate was higher in Td and Mp than in Sc Macabeo wines (Table 6). This would be due to the metabolic activity of autochthonous LAB, found in higher populations in these wines before *O. oeni* inoculation. Presumably due to the same reason, the development of autochthonous LAB, ethyl lactate was also high in all Cabernet Sauvignon wines at the end of AF. In fact, the increase of ethyl lactate in wine is associated to MLF metabolism (Liu, 2002).

No changes were observed in fusel alcohol concentration in the different conditions (Table 6) and also remained constant throughout the vinification process (Suppl. Table S2). In Macabeo and Cabernet Sauvignon, independently of whether non-*Saccharomyces* were used, wines clustered in general on the basis of MLF strategy (Figure 11).

Table 6. Concentrations of wine volatile compounds (mg/L) after AF grouped as family compounds. Σ, Sum; SCFA (propionic, butyric and valeric acids), MCFA (hexanoic, octanoic and decanoic acids), Ethyl esters of FA (ethyl hexanoate, ethyl octanoate, ethyl dodecanoate), Fusel alcohols (isobutanol, 1-butanol, 3-methyl-1-butanol, 1-hexanol, cis-3-hexen-1-ol, 2-phenylethanol, benzyl alcohol), Fusel alcohol acetates (isobutyl, isoamyl, hexyl and 2-phenylethanol acetates). Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. Values shown are the mean of triplicates ± SD. Statistics were calculated independently for each grape variety. n.d, not detected.

		Σ SCFA (mg/L)	Σ MCFA (mg/L)	Σ Ethyl esters of FA (mg/L)	Diethyl succinate (mg/L)	Ethyl lactate (mg/L)	Σ Fusel alcohols (mg/L)	Σ Fusel alcohol acetates (mg/L)
Macabeo	Sc	14.5 ± 0.7^{b}	16.3 ± 0.5^{a}	8.1 ± 0.1^{b}	n.d.^{b}	n.d.^{c}	121.5 ± 1.1^{a}	6.5 ± 0.2^{a}
	Td	9 ± 1.2^{c}	2 ± 0.4^{c}	9.2 ± 0.2^{a}	1.6 ± 0.1^{a}	53.4 ± 0.7^{a}	116 ± 0.4^{a}	2.1 ± 0.8^{c}
	Mp	19 ± 0.5^{a}	5.3 ± 0.1^{bc}	9.5 ± 02^{a}	n.d.^{b}	32.8 ± 13.5^{b}	115.3 ± 13.3^{a}	4.2 ± 0.1^{b}
Cabernet Sauvignon	Sc	n.d.^{c}	1.2 ± 0.2^{a}	3 ± 0.1^{a}	3.3 ± 0.7^{a}	81.9 ± 1.6^{a}	248.5 ± 92^{a}	3 ± 0.1^{a}
	Td	5.7 ± 1.8^{b}	0.6 ± 0.1^{b}	3.8 ± 0.6^{a}	4.6 ± 1^{a}	71.8 ± 0.9^{a}	265.4 ± 1.4^{a}	1.2 ± 0.2^{b}
	Mp	9.2 ± 0.8^{a}	n.d.^{c}	3.1 ± 0.1^{a}	2.9 ± 0.9^{a}	85.4 ± 18.6^{a}	252.4 ± 13.3^{a}	1.5 ± 0.8^{ab}

$^{a–c}$ Values are significantly different at $p \leq 0.05$. according to a Tukey HSD post-hoc comparison test.

The Macabeo wines that clustered in terms of MLF strategy were all similar with the exception of Mp-C wines, which presented a high concentration of ethyl esters, which differentiated them from Sc-C and Td-C wines (Figure 11, Suppl. Figure S1). The combination of strains used in Mp-C was clearly the one producing a profile of volatile compounds more different from the rest. It should also be mentioned that the Mp-S wines clustered together with the wines inoculated with *O. oeni* PSU-1 (Figure 11B). This was mainly due to the similar production of propionic and pentanoic acids (Suppl. Table S2). Despite the fact that in all wines with spontaneous MLF the main *O. oeni* genotype detected at the end of the fermentation was AB1 (Table 4), the different profile of volatile compounds in Mp-S wines might be due to the metabolism of diverse strains developing along MLF. Spontaneous fermentations, lacking the pressure of the massive inoculation of one strain, may allow a wider diversity of strains succession which can result in more unpredictable metabolic changes.

In contrast to white winemaking, in which spontaneous MLF presented the lowest concentrations of volatile compounds, in Cabernet Sauvignon this was the MLF strategy that resulted the most aromatic (Figure 11D). Despite the strain detected in

higher proportion in all Cabernet Sauvignon wines at the end of MLF belonged to IN1 genotype (Table 4), these wines showed differences in the aromatic profile and clustered according to the MLF inoculation strategy (Figure 11D). Even if the inoculated strain was not detected at the end of MLF, as in the case of CH11, it may have been present in the early stages of the fermentation contributing to define the aromatic profile.

In summary, the volatile composition of wines was modulated by non-*Saccharomyces* yeasts to produce different wines. However, the aromatic composition was homogenised after MLF, which was dependent on the *O. oeni* strain inoculated.

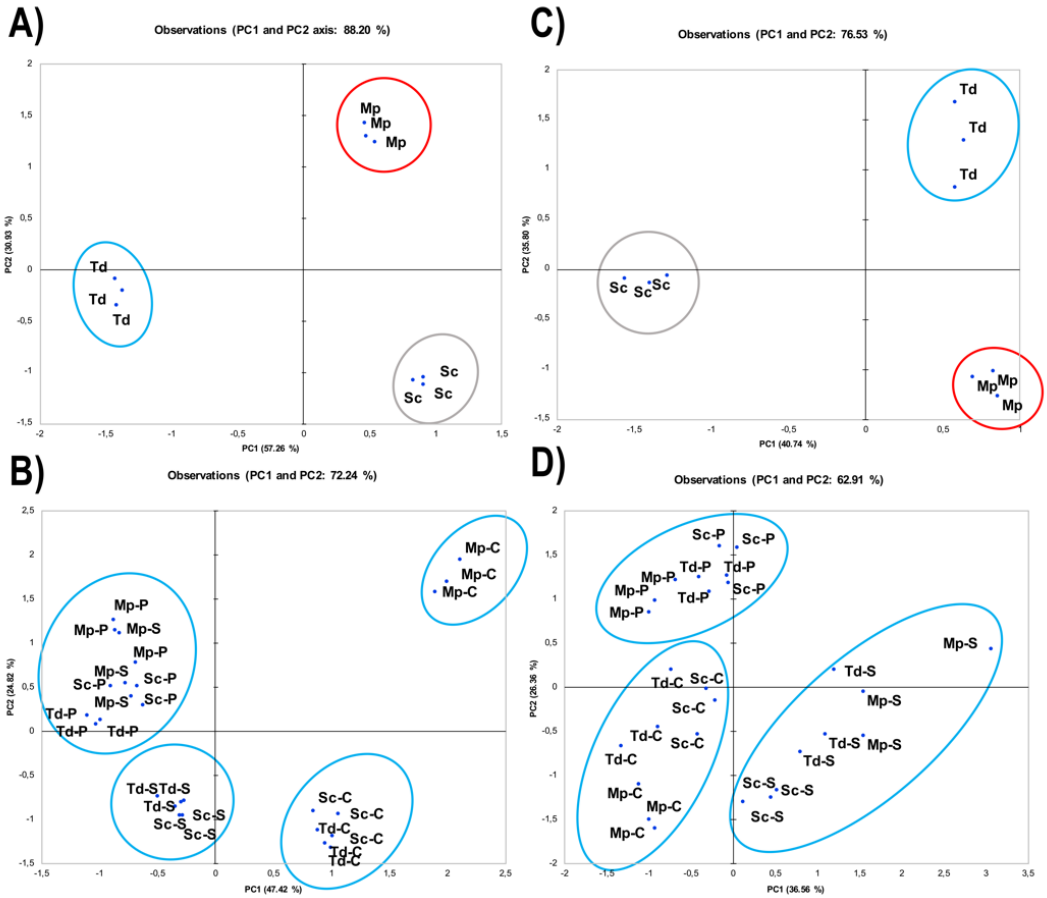

Figure 11. Principal component analysis (PCA) biplots of varimax rotated PCA for wine volatile composition in which observations are plotted. (A) Macabeo wines after AF. (B) Macabeo wines after MLF. (C) Cabernet Sauvignon wines after AF. (D) Cabernet Sauvignon wines after MLF. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

Colour

The colour parameters of both Cabernet Sauvignon and Macabeo wines were analysed, but no changes were observed in the latter. Due to the considerable chemical changes in MLF, mainly driven by the increase in pH, the colour parameters were affected in the red wine (Suppl. Figure S2). As expected, wines after AF had higher values of h*, C* and a*. Interestingly, colour parameters, after both AF and MLF, are grouped in terms of yeast inoculation strategy. Wines inoculated with a non-*Saccharomyces* belong to a cluster different from those inoculated with *S. cerevisiae* as sole starter (Suppl. Figure S2).

Polyphenolic compound content

The overall content of polyphenols in red Cabernet Sauvignon wines did not change during the vinification process. The total polyphenolic index (TPI) remained around 40 in all wines (data not shown). This low value was associated with a less effective colour extraction due to the method used to punch down in the small volume fermenters. Regarding to the anthocyanin concentration, Td wines after AF presented higher anthocyanin amount than Sc wines (Suppl. Figure S3). These differences were maintained after MLF disregarding the *O. oeni* strain used. Also, all Mp wines after MLF showed higher concentrations of anthocyanin than Sc wines. These results are in accordance with previous works describing incremented amounts of anthocyanin in wines inoculated with *T. delbrueckii* and *M. pulcherrima* when compared to wines inoculated only with *S, cerevisiae* (Escribano-Viana et al., 2019; Minnaar et al., 2015). No changes were observed in tannin concentrations.

Wine tasting

Cabernet Sauvignon sensory analysis did not result in concluding remarks. Wines could be clearly distinguished before and after MLF but there was not a clear clustering based on the inoculation strategy (data not shown). Macabeo wines were classified by tasters into three main clusters (Suppl. Figure S4): (i) wines after AF (red circle), (ii) wines after inoculated MLF (green circle) and (iii) wines after spontaneous MLF (blue circle). Wines after AF were the most acidic and oxidised. Wines with spontaneous

MLF had the most intense lactic character. Just one inoculated MLF wine is not included in the second cluster: Td-P. Interestingly, this was the wine which tasters preferred and described with the most moderate marks.

The wine tasting revealed that the most important changes in the chemical composition of the wines were not perceived by tasters. In this regard, the wine which was the most different in terms of volatile composition, Mp-C, was grouped in the inoculated wine cluster. The tasting data on acidity correlated with the pH values of the most acidic wines after AF (Table 5).

Sensory analysis was more variable in Macabeo wines, probably because fermentation was slower. Moreover, most of the chemical changes brought about by the different starter cultures were not noticed in the sensory evaluation of the resulting wines.

Conclusion

This paper reports novel research into the evaluation of the effect of two non-*Saccharomyces* on MLF in white and red winemaking under cellar conditions. The changes in wine composition was dependent on the type of winemaking and on the yeast strains used. Regarding the colour in red winemaking, wines inoculated with non-*Saccharomyces* showed higher concentrations of anthocyanin at the end of MLF than those inoculated only with *S. cerevisiae*. The aromatic profile of the wines was very dependent on the MLF strategy, highlighting the impact of *O. oeni* metabolic traits on the organoleptic characteristics. The inoculation of non-*Saccharomyces* yeasts caused longer AF in Macabeo wines. The MLF was faster in most of the wines inoculated with non-*Saccharomyces*, and the differences were more evident in Macabeo wines. The use of *M. pulcherrima* and *T. delbrueckii* resulted in lower SO_2 and MCFA concentrations at the end of the AF, offering more MLF friendly conditions than *S. cerevisiae* alone. The use of *T. delbrueckii* resulted in the fastest MLF in Macabeo wines and in the maximum *O. oeni* strain diversity in Cabernet Sauvignon wines. Altogether, *T. delbrueckii* metabolic fingerprint in wine seems to promote the development of *O. oeni* and improve MLF performance. Future research should

attempt to provide greater insight into the impact of different *T. delbrueckii* strains on MLF in different types of wine.

Acknowledgements

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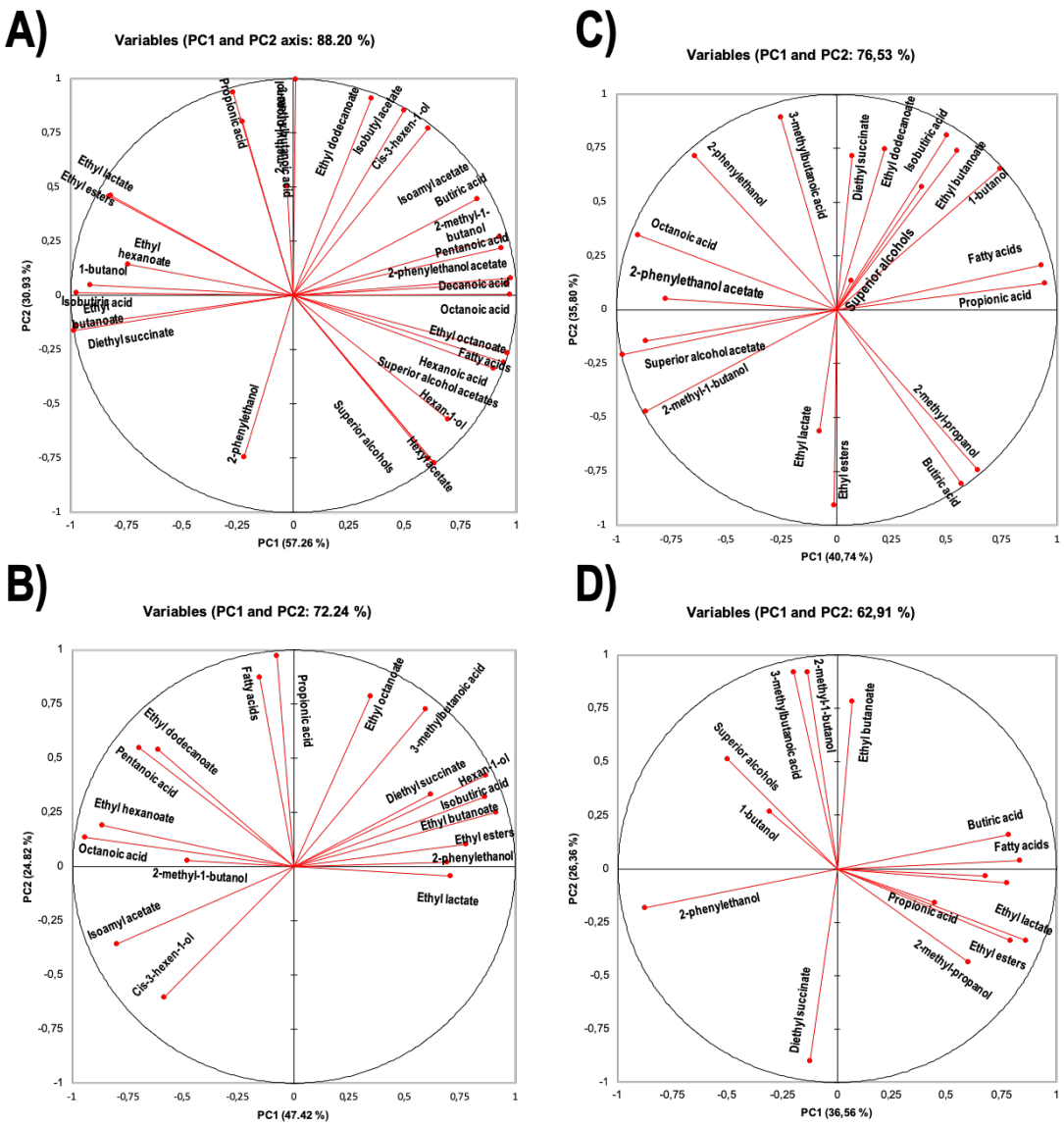
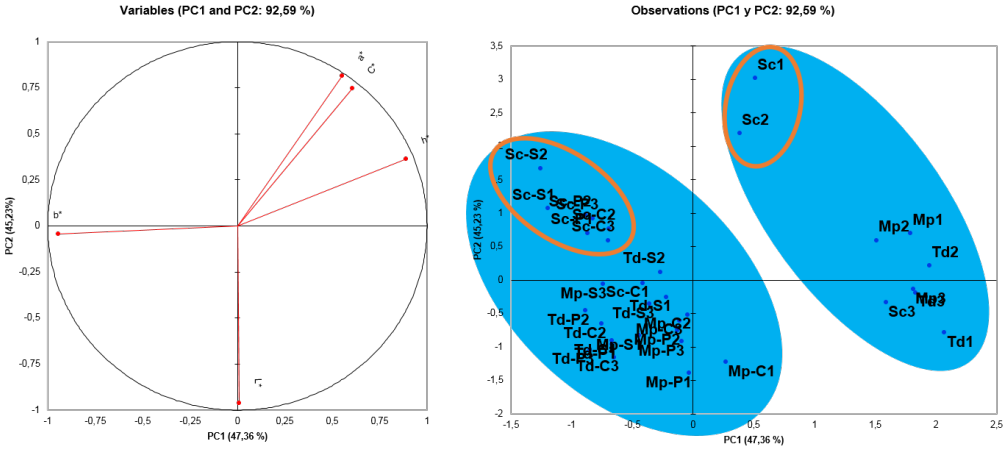
Supplementary Figures

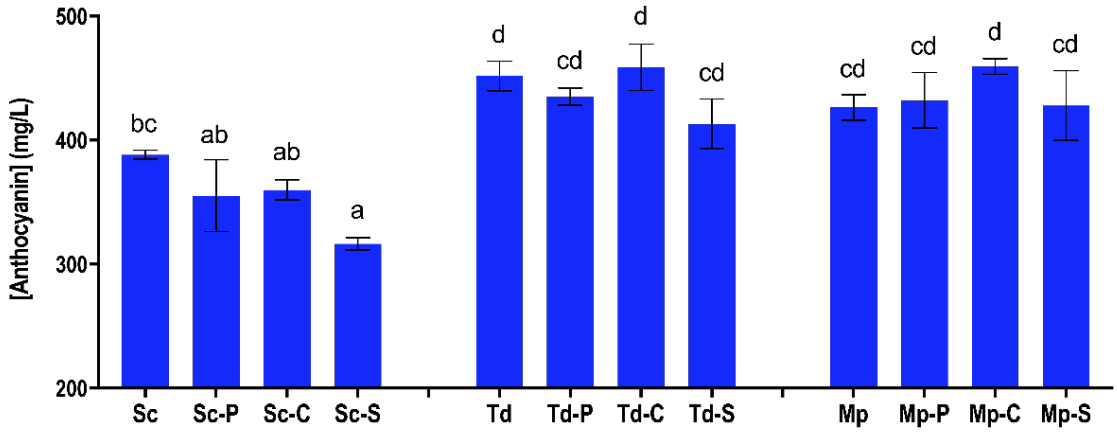
Supplementary Figure S1. Variable distribution of the principal component analysis (PCA) of varimax rotated PCA for wine volatile composition. (A) Macabeo wines after AF. (B) Macabeo wines after MLF. (C) Cabernet Sauvignon wines after AF. (D) Cabernet Sauvignon wines after MLF.



Suppl. Figure S2. Principal component analysis (PCA) biplots of varimax rotated PCA for Cabernet Sauvignon wine colour parameters in which variables and observations are separately plotted. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

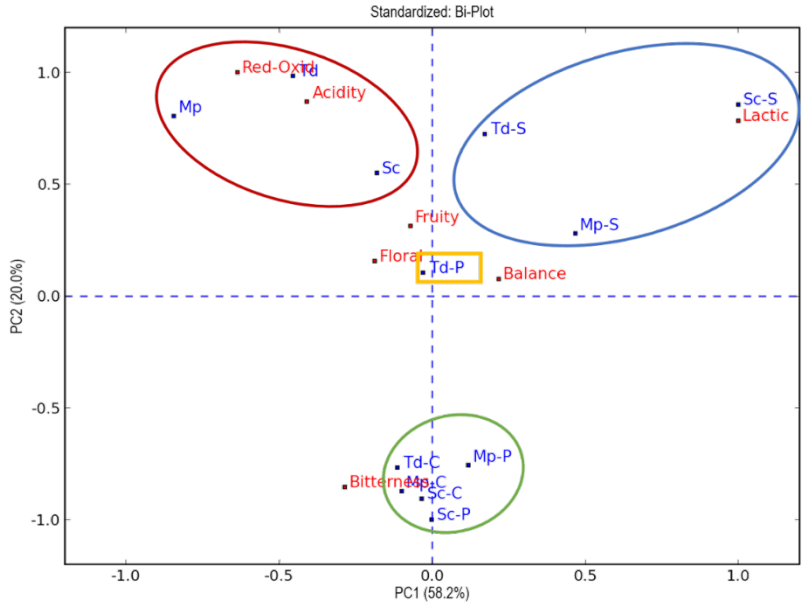


Suppl. Figure S3. Anthocyanin concentration (mg/L) of Cabernet Sauvignon wines. Values shown are the mean of triplicates ± SD. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.



* a–d. values are significantly different at p ≤ 0.05. according to a Tukey HSD post-hoc comparison test.

Suppl. Figure S4. Principal component analysis (PCA) biplot of varimax rotated PCA for wine sensory analysis of Macabeo wines. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated. Wines are grouped as (red) wines after AF, (green) wines after inoculated MLF and (blue) wines after spontaneous MLF) with the exception of Td-P wine.



Supplementary Tables

Suppl. Table S1. Oenological parameters of wines after alcoholic and malolactic fermentations. Values shown are the means of triplicates ± SD. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii* - *S. cerevisiae* and *M. pulcherrima* - *S. cerevisiae* fermented wines, respectively. P, C, and S refer to the MLF strategy were O. oeni PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

	Glucose+ fructose (g/L)	D-lactic acid (g/L)	NOPA (mg/L)	NH_4 (mg/L)	Succinic acid (mg/L)
Macabeo					
Sc	0.62 ± 0.38	0.13 ± 0.01	7.94 ± 1.18	3.33 ± 2.05	257.38 ± 1.75
Sc-P	n.d.	0.15 ± 0.01	10.35 ± 1.29	2 ± 2.16	
Sc-C	n.d.	0.19 ± 0.01	8.34 ± 1.44	4 ± 0.82	
Sc-S	n.d.	0.17 ± 0	4.05 ± 1.32	1.33 ± 1.25	
Td	2.03 ± 0.17	0.2 ± 0.01	9.85 ± 1.57	2 ± 2.83	263.33 ± 2.31
Td-P	n.d.	0.26 ± 0	10.74 ± 1.32	2.67 ± 1.25	
Td-C	n.d.	0.24 ± 0.01	10.89 ± 1.36	1.33 ± 1.89	
Td-S	n.d.	0.25 ± 0	7.27 ± 0.36	4 ± 2.83	
Mp	0.28 ± 0.05	0.12 ± 0	8.25 ± 1.32	2.67 ± 2.05	258.82 ± 1.89
Mp-P	n.d.	0.24 ± 0.01	4.38 ± 0	n.d.	
Mp-C	n.d.	0.23 ± 0.01	4.45 ± 0.82	3.33 ± 0.94	
Mp-S	n.d.	0.2 ± 0	3.78 ± 0.32	0.33 ± 0.47	
Cabernet Sauvignon					
Sc	0.19 ± 0.02	0.27 ± 0.07	23.17 ± 2.6	14.67 ± 7.57	192.57 ± 23.39
Sc-P	n.d.	0.42 ± 0.01	23.85 ± 1.09	5 ± 2	
Sc-C	n.d.	0.43 ± 0.01	21.87 ± 3.89	4.67 ± 2.08	
Sc-S	n.d.	0.22 ± 0.08	4.88 ± 1.3	n.d.	
Td	n.d.	0.21 ± 0.02	16.39 ± 5.8	18 ± 4.36	234.31 ± 6.73
Td-P	n.d.	0.42 ± 0.04	21.59 ± 4.08	7.33 ± 3.79	
Td-C	n.d.	0.44 ± 0.01	23.93 ± 3.75	8.33 ± 2.08	
Td-S	n.d.	0.33 ± 0.02	4.94 ± 0.54	4.33 ± 2.31	
Mp	n.d.	0.23 ± 0.03	20.99 ± 1.98	12.33 ± 3.21	215.22 ± 8.93
Mp-P	n.d.	0.41 ± 0.01	2.23 ± 0.12	n.d.	
Mp-C	n.d.	0.43 ± 0.01	2.29 ± 1.1	3.67 ± 3.51	
Mp-S	n.d.	0.39 ± 0.01	2.89 ± 1.17	n.d.	

Suppl. Table S2. Concentrations of wine volatile compounds (mg/L). Values shown are the mean of triplicates ± SD. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. PSU, CH11 and Spontaneous refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

	Sc	Td	Mp	Sc-P	Sc-C	Sc-S	Td-P	Td-C	Td-S	Mp-P	Mp-C	Mp-S
Macabeo wines												
Propionic acid	6.23 ± 0.02	8.35 ± 1.84	9.69 ± 0.05	8.5 ± 0.68	n.d.	n.d.	6.26 ± 0.65	n.d.	n.d.	11.12 ± 0.78	12.28 ± 0.49	8.73 ± 0.86
2-methylpropanoic acid	2 ± 0.08	4.44 ± 0.27	2.47 ± 0.28	1.88 ± 0.72	3.31 ± 0.25	2.68 ± 0	1.78 ± 0.09	3.79 ± 0.08	2.75 ± 0.49	2.45 ± 0.76	6.69 ± 0.09	2.66 ± 0.47
Butanoic acid	5.76 ± 2.37	n.d.	8.74 ± 0.44	n.d.	2.48 ± 0.17	n.d.	n.d.	n.d.	3.31 ± 0.8	8.38 ± 1.59	n.d.	5.28 ± 0.44
3-methylbutanoic acid	0.97 ± 0.22	1.13 ± 0.12	1.36 ± 0.46	1 ± 0.06	0.81 ± 0.03	0.78 ± 0.14	0.9 ± 0.12	0.95 ± 0.1	0.98 ± 0.04	1.44 ± 0.31	2.94 ± 0.08	0.96 ± 0.1
Pentanoic acid	0.51 ± 0.05	n.d.	0.56 ± 0.12	0.44 ± 0.06	n.d.	n.d.	0.35 ± 0.05	n.d.	n.d.	0.56 ± 0.12	n.d.	0.46 ± 0.07
Hexanoic acid	10.49 ± 0.52	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Octanoic acid	4.42 ± 0.07	2.02 ± 0.43	4.04 ± 0.15	4.63 ± 0.01	1.95 ± 0.2	4.34 ± 0.06	5.36 ± 0.15	1.65 ± 0.07	4.21 ± 0.19	4.48 ± 0.07	1.64 ± 0.16	4.41 ± 0.41
Decanoic acid	1.43 ± 0.23	n.d.	1.27 ± 0.2	0.75 ± 0.01	0.72 ± 0.01	0.79 ± 0.06	0.82 ± 0.03	0.86 ± 0.03	0.83 ± 0.03	1.49 ± 0.54	1.24 ± 0.2	0.9 ± 0.07
Ethyl butanoate	2.65 ± 0.1	4.41 ± 0.11	2.67 ± 0.21	2.45 ± 0.07	5.56 ± 0.15	2.8 ± 0.05	2.88 ± 0.21	3.78 ± 0.21	2.51 ± 0.27	2.48 ± 0.33	7.49 ± 0.04	2.73 ± 0.25
Ethyl hexanoate	1.81 ± 0.19	2.26 ± 0.16	1.96 ± 0.13	1.05 ± 0.03	n.d.	0.88 ± 0.13	1.02 ± 0.1	n.d.	1.13 ± 0.36	1.03 ± 0.06	n.d.	1.67 ± 0.29
Ethyl lactate	n.d.	53.37 ± 0.67	32.82 ± 13.49	32.53 ± 0.63	68.4 ± 6.14	54.45 ± 0.58	8.49 ± 0.92	61.49 ± 0.2	49.65 ± 2.27	40.99 ± 0.07	72.8 ± 12.25	63.43 ± 1.3
Ethyl octanoate	3.69 ± 0.06	1.8 ± 0.03	2.9 ± 0.06	n.d.	0.41 ± 0.58	n.d.	n.d.	0.64 ± 0.91	n.d.	3.15 ± 0.07	4.4 ± 0.82	2.68 ± 0.17
Diethyl succinate	n.d.	1.64 ± 0.08	n.d.	1.02 ± 0.06	1.35 ± 0.37	n.d.	n.d.	1.48 ± 0.01	n.d.	n.d.	5.15 ± 0.31	n.d.
Ethyl dodecanoate	n.d.	0.68 ± 0.09	1.94 ± 0.02	2.38 ± 0.42	1.25 ± 0.38	1.74 ± 0.11	2.29 ± 0	0.94 ± 0.16	1.83 ± 0.08	2.36 ± 0.36	1.75 ± 0.1	1.83 ± 0.23
2-methyl-propanol	13.45 ± 0.96	23.13 ± 1.75	29.26 ± 1.77	12.32 ± 0.65	26.77 ± 0.4	26.51 ± 3.97	13.05 ± 0.06	21.65 ± 0.14	26.52 ± 3.1	13.18 ± 0.79	24.15 ± 0.09	30.63 ± 1.02
1-butanol	n.d.	1.44 ± 0.11	0.33 ± 0.46	1.29 ± 0.21	1.4 ± 0.38	n.d.	1.01 ± 0.13	1.75 ± 0.04	n.d.	n.d.	0.67 ± 0.95	0.93 ± 0.13
3-mehyl-1-butanol	71.77 ± 0.85	65.09 ± 1.18	72.83 ± 0.66	66.36 ± 1.37	67 ± 3.36	79.57 ± 0.6	72.94 ± 2.02	63.62 ± 0.22	75.01 ± 3.57	71.78 ± 1.31	66.29 ± 10.8	80.74 ± 0.01
1-hexanol	0.75 ± 0.02	0.41 ± 0.02	0.48 ± 0.12	0.7 ± 0.08	0.81 ± 0.25	0.39 ± 0.02	0.76 ± 0.07	0.8 ± 0.13	0.4 ± 0.08	0.54 ± 0.05	1.48 ± 0.77	0.37 ± 0.04
cis-3-hexen-1-ol	0.66 ± 0.01	0.64 ± 0	0.76 ± 0.01	0.57 ± 0.07	0.51 ± 0.09	0.61 ± 0.05	0.59 ± 0.07	0.72 ± 0	0.51 ± 0.07	0.6 ± 0.1	n.d.	0.52 ± 0.02
2-phenylethanol	46.82 ± 0.79	45.69 ± 1.33	33.86 ± 7.66	43.69 ± 8.59	56.05 ± 4.55	30.88 ± 1.8	43.31 ± 0.16	56.66 ± 9.51	29.62 ± 1.01	41.34 ± 1.26	57.42 ± 2.2	25.93 ± 0.81
2-methylpropyl acetate	n.d.	n.d.	1.03 ± 0.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.27 ± 0.52
3-methylbutyl acetate	0.92 ± 0.01	0.68 ± 0.02	1.47 ± 0.12	0.71 ± 0.01	0.77 ± 0.25	0.96 ± 0.01	1.04 ± 0.02	0.71 ± 0.05	1.01 ± 0.11	0.99 ± 0.16	n.d.	1.21 ± 0.17

Hexyl acetate	2.61 ± 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-phenylethanol acetate	2.5 ± 0.26	0.51 ± 0.02	2.32 ± 0.16	1.94 ± 0.26	0.47 ± 0.02	0.99 ± 0.03	2.35 ± 0.02	1.03 ± 0.16	0.99 ± 0.02	0.31 ± 0	2.57 ± 1.19	0.35 ± 0.04
Cabernet Sauvignon wines												
Propionic acid	n.d.	5.74 ± 1.81	5.62 ± 0.88	5.7 ± 1.19	3.86 ± 0.53	5.76 ± 0.22	4.41 ± 0.3	7.27 ± 0.15	7.72 ± 0.68	6.32 ± 0.57	6.24 ± 1.12	8.52 ± 0.99
2-methylpropanoic acid	2.53 ± 0.75	5.06 ± 0.42	2.81 ± 0.04	2.24 ± 0.11	5.3 ± 1.32	2.06 ± 0.2	2.63 ± 0.17	4.47 ± 0.12	3.38 ± 1.18	3.58 ± 0.37	4.85 ± 0.31	3.74 ± 0.48
Butanoic acid	n.d.	n.d.	3.54 ± 0.06	3.05 ± 0.92	n.d.	2.64 ± 0.22	3.53 ± 0.49	n.d.	5.29 ± 0.26	n.d.	n.d.	4.19 ± 0.15
3-methylbutanoic acid	1.11 ± 0.02	1.23 ± 0.16	0.84 ± 0.05	2.19 ± 0.15	1.37 ± 0.04	0.82 ± 0.06	1.69 ± 0.24	1.34 ± 0.16	1.17 ± 0.33	1.78 ± 0.02	0.98 ± 0.1	1.18 ± 0.11
Hexanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	3.32 ± 0.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Octanoic acid	1.25 ± 0.24	0.57 ± 0.11	n.d.	n.d.	0.66 ± 0.07	0.69 ± 0	1.28 ± 0.07	0.75 ± 0.02	0.85 ± 0.08	1.28 ± 0.11	0.76 ± 0.1	0.91 ± 0.14
Ethyl butanoate	2.43 ± 0.14	2.8 ± 0.44	2.53 ± 0.15	2.86 ± 0.24	2.44 ± 0.3	2.09 ± 0.19	2.7 ± 0.2	2.62 ± 0.32	2.25 ± 0.33	2.72 ± 0.18	2.16 ± 0.22	2.59 ± 0.64
Ethyl lactate	81.89 ± 1.65	71.83 ± 0.88	85.35 ± 18.57	89.9 ± 2.43	74.08 ± 3.86	107.72 ± 2.29	77.04 ± 1.12	79.52 ± 0.43	115.14 ± 13.08	65.24 ± 2.67	85.24 ± 9.91	109.71 ± 9.53
Diethyl succinate	3.34 ± 0.65	4.58 ± 1.02	2.85 ± 0.9	1.65 ± 0.08	2.78 ± 0.08	3.36 ± 0.01	1.46 ± 0.16	3.05 ± 0.18	2.94 ± 0.17	1.55 ± 0	4.56 ± 0.42	2.36 ± 0.26
Ethyl dodecanoate	0.63 ± 0.01	0.96 ± 0.32	0.58 ± 0.06	0.83 ± 0.26	0.49 ± 0.04	n.d.	0.55 ± 0.06	0.67 ± 0.07	0.53 ± 0.05	0.8 ± 0.13	0.81 ± 0.25	0.55 ± 0.18
2-methyl-propanol	36.3 ± 3.38	39 ± 4.08	56.95 ± 0.52	36.26 ± 2.87	42.19 ± 0.14	45.33 ± 3.29	34.37 ± 1.39	44.53 ± 2.04	45.74 ± 1.45	35.75 ± 1.73	35.84 ± 3.51	47.56 ± 2.06
1-butanol	1.19 ± 0.02	1.97 ± 0.04	1.5 ± 0.04	1.45 ± 0.06	1.86 ± 0.35	n.d.	1.53 ± 0.11	2.09 ± 0.23	1.16 ± 1.03	1.43 ± 0.08	1.68 ± 0.27	1.5 ± 0.01
2-methyl-1-butanol	140.4 ± 1.63	102.79 ± 2.37	118.68 ± 0.89	134.35 ± 8.91	104.88 ± 2.66	104.95 ± 1.38	128.44 ± 1.07	108 ± 6.69	109.55 ± 1.11	129.08 ± 4.5	97.23 ± 2.72	105.35 ± 2.86
Phenylmethanol	1.3 ± 0.22	0.88 ± 0.18	0.82 ± 0.27	0.9 ± 0.16	1.09 ± 0.28	1 ± 0.17	0.96 ± 0.09	1.21 ± 0.01	1.16 ± 0.03	1.1 ± 0.06	1.52 ± 0.06	1.28 ± 0.32
2-phenylethanol	134 ± 18.72	124.09 ± 3.18	60.04 ± 1.54	87.21 ± 1.41	84.27 ± 3.16	85.42 ± 6.15	86.55 ± 5.04	108.6 ± 4.39	75.45 ± 3.11	88.62 ± 8.53	103.81 ± 0.87	59.92 ± 1.17
2-methylpropyl acetate	0.46 ± 0.08	0.98 ± 0.13	0.55 ± 0.03	0.49 ± 0.12	0.63 ± 0.04	0.52 ± 0.08	0.6 ± 0.15	n.d.	0.54 ± 0.01	0.53 ± 0.01	0.89 ± 0.43	0.53 ± 0.02
2-phenylethanol acetate	2.54 ± 0.24	n.d.	0.37 ± 0.1	n.d.	0.39 ± 0	n.d.	n.d.	n.d.	0.49 ± 0.13	n.d.	0.14 ± 0.25	1.69 ± 1.23
4-ethyl-phenol	n.d.	n.d.	n.d.	n.d.	0.67 ± 0.13	n.d.	n.d.	0.87 ± 0.11	n.d.	n.d.	1.13 ± 0.23	n.d.
4-ethyl-guayacol	n.d.	n.d.	0.55 ± 0.04	0.96 ± 0.13	n.d.	0.21 ± 0.36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

CHAPTER I: 2

Torulaspora delbrueckii promotes malolactic fermentation in high polyphenolic red wines

Aitor Balmaseda[a], Nicolas Rozès[b], Albert Bordons[a], Cristina Reguant[a,*]

* Corresponding author: cristina.reguant@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

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Abstract

Using *Torulaspora delbrueckii* as starter culture for alcoholic fermentation (AF) is a current trend for enhancing the quality of red wines. As red winemaking usually requires subsequent malolactic fermentation (MLF), the compatibility of this yeast and *Oenococcus oeni* is a key factor for a successful fermentative process. In this work we study the interactions of *T. delbrueckii* and *O. oeni* in wines from grapes with different degrees of maturity. The results showed higher total polyphenolic index (TPI) values in *T. delbrueckii* wines. Moreover, the aromatic characteristics were improved in these wines, compared to the wines inoculated only with *Saccharomyces cerevisiae*. There was also a reduction in some inhibitor compounds for *O. oeni*, for instance medium chain fatty acids, as a result of the fermentation with this non-*Saccharomyces.* Overall, the use of *T. delbrueckii* resulted in better MLF performances.

Keywords

Non-*Saccharomyces*, *Torulaspora delbrueckii*, malolactic fermentation, *Oenococcus oeni*, wine

Introduction

Yeasts and lactic acid bacteria (LAB) are the most important microorganisms for determining the quality of wine through their metabolism and interactions (Petruzzi et al., 2017). *Saccharomyces cerevisiae* is the predominant yeast species in the final stages of alcoholic fermentation (AF). High yeast diversity is found in the first stages of AF, including species of *Hanseniaspora*, *Torulaspora* and *Metschnikowia*. These non-*Saccharomyces* lose their viability when the ethanol concentration begins to increase (Vilela, 2019). LAB, mainly *Oenococcus oeni*, play an important role in the winemaking process through malolactic fermentation (MLF), by decarboxylating L-malic acid into L-lactic acid, improving wine quality and microbial stability (Davis et al., 1985; Lonvaud-Funel, 1999).

Some non-*Saccharomyces*, such as *T. delbrueckii*, are a current winemaking trend. This yeast can be found in late AF due to its high metabolic activity under winemaking conditions and also due to its resistance to ethanol and SO_2 (Benito, 2018a). Moreover, *T. delbrueckii* and *S. cerevisiae* are genetically close (Masneuf-Pomarede et al., 2016).

The microbial community and the specific inoculated strains play an important role in the organoleptic profile of wine. Indeed, some strains can help in the extraction of aromas and polyphenols from grape skins, which improves both aroma complexity and colour. This is currently of great interest because climate change means that grapes must be harvested with a low secondary metabolite concentration to meet a compromise with the sugar concentrations (Ubeda et al., 2020). Typical red wines have a concentration of around 500 mg/L of anthocyanins (mainly monomeric anthocyanins), which leads to larger pigments with higher stability during AF, MLF and maturation (Watrelot and Norton, 2020). The standard concentration of tannins in red wines of *Vitis vinifera* cultivars is 1-4 g/L (Asenstorfer et al., 2001). In addition, high polyphenolic wines can cause difficulties for *O. oeni* to perform MLF (Reguant et al., 2000).

T. delbrueckii is proposed as a tool for modulating the wine aromatic profile (Belda et al., 2015). Its metabolic activity helps to release terpene aromas such as α-terpineol and linalool (Čuš and Jenko, 2013). Its use is related to an enhancement in the fruity character of wines (Morata et al., 2020). Moreover, it can help to enhance red wine

colour (Escribano-Viana et al., 2019), reduce the ethanol content (Belda et al., 2017c; Contreras et al., 2014), decrease the fatty acid concentration (Benito, 2018a), and increase mannoprotein and glycerol contents (Belda et al., 2015; González-Royo et al., 2015) in mixed fermentation together with *S. cerevisiae*. Altogether, *T. delbrueckii* is reported as a stimulating yeast for MLF (Balmaseda et al., 2018; Martín-García et al., 2020). In this final point, the compatibility and microbial interaction mediated consequences of *T. delbrueckii* and *O. oeni* has been studied recently in white and red winemaking (**Chapter I: 1**).

To better understand the suitability of *T. delbrueckii* for red winemaking and its effect on MLF, we studied these microbial interactions in Merlot winemaking with two grape maturity levels using two strains of this yeast species in sequential inoculation with *S. cerevisiae*. We compared our results with a wine fermented only with *S. cerevisiae*. We monitored MLF in inoculated fermentations, evaluating two *O. oeni* strains, and also in spontaneous MLF. This work offers novel data regarding the impact of the use of *T. delbrueckii* on wine polyphenolic content along the red winemaking process. The use of different *T. delbrueckii* and *O. oeni* strains provided information about the metabolic traits affecting wine composition that may be species or strain dependent.

Materials and methods

Microorganisms

AF was carried out using three yeast strains: *T. delbrueckii* Biodiva (TdB) (Lallemand Inc., Montréal, Canada), *T. delbrueckii* Viniferm (TdV) (Agrovin, Alcázar de San Juan, Spain) and *S. cerevisiae* Lalvin-QA23 (Sc) (Lallemand Inc.). Yeasts were stored as active dry yeasts at 4 ºC. Two strains of *O. oeni* were used: PSU-1 (ATCC BAA-331) and Viniflora-CH11 (Chr. Hansen AS, Hoersholm, Denmark), which were kept on MRSmf plates (Margalef-Català et al., 2017) and stored at 4 ºC.

Fermentation trials

Fermentations were carried out with red Merlot grapes from a vineyard in Vilafranca del Penedès (Catalonia, Spain). The vineyard was harvested before the optimal maturity level (Merlot 1) and at the optimal maturity level 10 days after (Merlot 2) during the 2019 vintage. The maturity of Merlot 2 resulted in a less acid must and increased YAN (yeast assimilable nitrogen) concentration, while the other parameters were similar to Merlot 1 (Suppl. Table S3). Grapes and the resulting musts were processed as in **Chapter I: 1**. Briefly, about 100 kg of red grapes were manually harvested each time and then processed in the experimental cellar of *Rovira i Virgili* University.

Alcoholic fermentations were carried out with each *T. delbrueckii* strain and *S. cerevisiae* was inoculated after 48h. Fermenters were supplemented with nutrients (0.4 g/L Nutrient Vit NatureTM, Lallemand Inc.) together with *S. cerevisiae* inoculation. Each yeast was inoculated for a population of $2.5 \cdot 10^6$ cells/mL with active dry yeast after rehydration with water following the manufacturer's instructions. There was also a control fermentation with *S. cerevisiae* as a sole starter (Sc). All fermentations were performed in triplicate. Samples of 6 mL were taken every 48h to monitor the density decrease and yeast population evolution. YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac Química SLU, Castellar del Vallès, Spain) was used to calculate the total number of yeast cells, and lysine agar medium (Oxoid LTD., England) for quantification of non-*Saccharomyces* yeasts (Wang et al., 2016), after incubation at 28 ºC for 48h. AF was considered finished when the sugar concentration was below 2 g/L. Fermentations were carried out at 27 ºC. Fermenting must was manually punched down every 48 hours during AF. Grape skins were always submerged thanks to a flat strainer used as a stopper in the fermenter.

After AF, wines were pressed and transferred to another container, cooled for 5 days and decanted. Then, wine samples were centrifugated and stored at -20 ºC. Later, equal volumes of each triplicate (0.5 L) were mixed and sulphited (10 mg/L $K_2S_2O_5$) in two 0.75 L bottles, which were stored at 4 ºC until wine tasting. The residual volume of the mixed wines was supplemented with L-malic acid for a concentration of 2 g/L. Then, the pH was corrected to the value before L-malic acid addition. Adjusted wines

were inoculated with two *O. oeni* strains, each in 1 L flasks at 20 ºC for a population of $2 \cdot 10^7$ cells/mL. In addition, a spontaneous MLF was followed. These fermentations were also carried out in triplicate. Samples were taken every 24h to monitor L-malic acid and the bacterial population. Samples were plated on MRSmf supplemented with nystatin (100 mg/L), sodium azide (25 mg/L) and tomato juice (100 mL/L, Aliada, Madrid, Spain), and incubated at 27 ºC in a 10 % CO_2 atmosphere for 7-15 days. MLF was considered finished when the L-malic acid was below 0.05 g/L. After AF and MLF, wines for tasting were sulphited (25 mg/L $K_2S_2O_5$).

Yeast and bacterial identification

Yeast identification

Twenty-five colonies were randomly selected for yeast identification isolated from must before the first inoculation, must before inoculating *S. cerevisiae* (48h) and wine at the end of AF (density below 995 g/L and residual sugars below 2 g/L). Isolate species were identified based on the amplicon size of the ITS-5.8S rDNA region (Esteve-Zarzoso et al., 1999).

LAB identification and strain typing of *Oenococcus oeni*

Colonies (10 from inoculated MLF to confirm the imposition of the inoculated strain and 20 from spontaneous MLF to evaluate the strain diversity) were randomly selected for LAB identification from MRSmf plates at the end of MLF. The identification of LAB species and strain typing of *O. oeni* were performed as described in Balmaseda et al. (2021). DNA was extracted with a High Pure PCR Template Preparation Kit (Roche, Barcelona, Spain). Briefly, LAB isolates with a cocci morphology were confirmed to be *O. oeni* by the species-specific PCR (Zapparoli et al., 1998). Non-*Oenococcus* isolates were identified with the 16S-ARDRA method and *Mse*I digestion according to Rodas et al. (2003). Isolates identified as *O. oeni* were typed by the multilocus variable number tandem repeat (VNTR) (Claisse and Lonvaud-Funel, 2014).

General oenological analytical parameters

Concentrations of sugars (glucose and fructose), L-malic acid, acetic acid, glycerol, D- and L-lactic, NH_4, primary ammonium nitrogen (NOPA), total and free SO_2 and citric acid were determined with a Miura One Multianalyzer (TDI, Barcelona, Spain) using enzymatic kits from TDI and Biosystems S.A. (Barcelona, Spain). Acetaldehyde and succinic acid were determined using the corresponding assay kits K-ACHYD and K-SUCC (Megazyme, Wicklow, Ireland), respectively. pH was determined using a Crison micro pH 2002 pH-meter (Barcelona, Spain) and alcoholic degree was determined by ebulliometry (Electronic ebulliometer uEBU6576, GabSystem) following the methods of the Compendium of International Methods of Analysis of Musts and Wines (OIV, 2009).

Analyses of volatile compounds

Wine samples (10 mL) were taken after AF and MLF. The volatile compounds were liquid/liquid extracted with 0.4 mL dichloromethane and 2.5 g $(NH_4)_2SO_4$ using 4-methyl-2-penthanol (0.8 g/L) and heptanoic acid (0.7 g/L) as internal standards following Ortega et al. (2001). After 90 min agitation at room temperature and centrifugation (5,080 g, 5 min), 2 µL of the organic phase was injected in split mode (10:1, 30 mL/min) into a gas chromatography HP-FFAP (30 m × 0.25 mm × 0.25 µm , Agilent Technologies, Böbligen, Germany). The temperature of the program was as follows: 35 ºC during 5 min, increased 3 ºC/min to 200 ºC, then 8 ºC/min to 220 ºC. The temperature of the injector and detector were 180 ºC and 280 ºC respectively. The gas carrier was He at 3 mL/min. Aromatic volatile compounds were identified and quantified by comparison with standards.

Colour parameters and phenolic characterisation

The colour of wine samples was analysed directly in a quartz cuvette with a 1 mm optical length based on the method of Glories (1984). CIELab coordinates: lightness (L), chroma (C), hue (h), red-greenness (a) and yellow-blueness (b) were determined according to Ayala et al. (1997) and data processing was performed with the MSCV software.

The phenolic composition was analysed in terms of the total polyphenol index (TPI), tannin concentration and anthocyanin concentration. TPI was analysed by measuring the 280 nm absorbance of a 1:100 dilution of wines with a spectrophotometer, using a 10 mm quartz cuvette and multiplying the absorbance value by 100 as described by Ribéreau-Gayon et al. (2006). Tannin concentration was determined based on the Bate-Smith method (Ribéreau-Gayon and Stonestreet, 1966) with some modifications (Vignault et al., 2018).

Wine tasting

Sensory analyses were performed after AF and MLF. Triplicates were blended for simplifying the analysis. Wines were evaluated by at least 12 tasters, considered as experts, from the Oenology Faculty of Rovira i Virgili University. The experts were given 20 mL of wine in dark glasses to avoid subjectivity due to the colour of the samples.

Samples were randomly numbered with 3-digit codes. Wines were served anonymously according to a Latin square of Williams design to avoid the range and carry-over effect.

Triangle tests were performed to evaluate differences between the produced wines. In addition, tasters were asked to write down their preference in each sequence. In addition to triangular tests, a classification test was performed. We selected some wines with distinct classifications and correctly assigned on triangle tests. Tasters were asked to classify the wines in terms of increasing intensity for three attributes: red fruit, lactic character and astringency.

Statistical analysis

The statistical software XLSTAT version 2020.1.1.64570 (Addinsoft, Paris, France) was used. The data obtained were submitted to one-way ANOVA with a subsequent analysis using the Tukey test, with a confidence interval of 95%. Results were considered significant when p-value ≤ 0.05. Principal Component Analysis (PCA) was also performed with the same statistical software to determine differences between the wines. The level of significance of sensory triangle tests was determined following

Jackson's method (Jackson, 2002). The sensorial classification test was analysed based on the Friedman test with a significance level of p-value ≤ 0.05 (Olkin et al., 2015).

Results and discussion

Fermentation performance

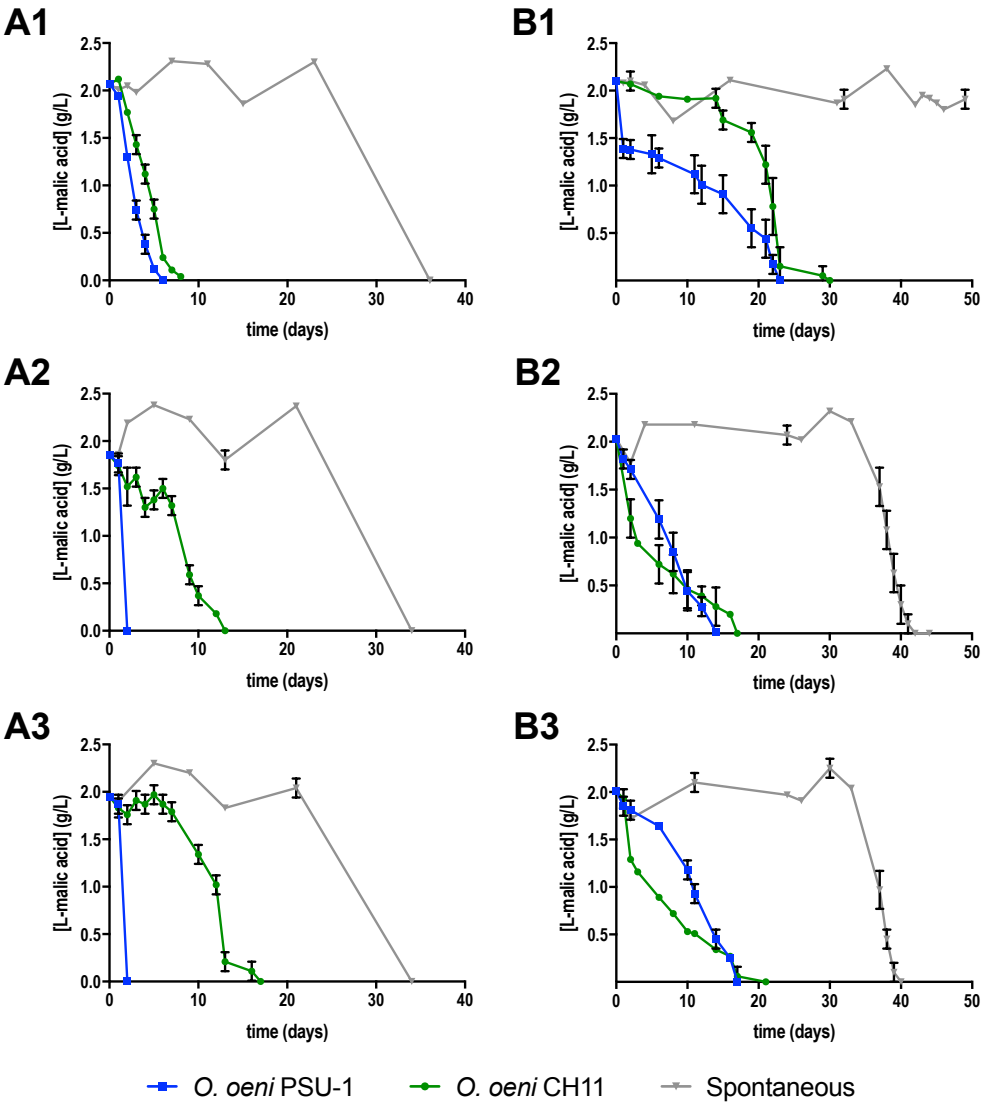

Figure 12. Evolution of malolactic fermentation after AF by monitoring the L-malic acid consumption. Left: Merlot 1 wines fermented with *S. cerevisiae* (A1), *T. delbrueckii* Biodiva-*S. cerevisiae* (A2), *T. delbrueckii* Viniferm-*S. cerevisiae* (A3). Right Merlot 2 wines fermented with *S. cerevisiae* (B1), *T. delbrueckii* Biodiva-*S. cerevisiae* (B2), *T. delbrueckii* Viniferm-*S. cerevisiae* (B3).

Wine AF was very quick in both Merlot grape musts at two different maturity levels. In both cases *S. cerevisiae* fermented wines finished AF in 8 days (Table 7). Little delay was observed in the wines fermented sequentially with *T. delbrueckii* and *S. cerevisiae*:

AF finished in 10 days in Merlot 1 and 12 days in Merlot 2 (Table 7). The observed AF extension in sequential inoculations is a common behaviour due to competition events between the inoculated fermenting yeasts (Belda et al., 2015; **Chapter I: 1**). Basically, the AF took longer because the yeasts needed more time to dry the wines (glucose + fructose < 2 g/L). The difference in the sugar consumption rate was not very remarkable (Table 7) as only a significant difference was observed in Sc wines regarding TdB and TdV wines with Merlot 2 must. The consumption rates of the AF (Table 7) were higher in Merlot 2 wines, probably due to higher nitrogen available (Suppl. Table S3) in this more mature grape must (Ali et al., 2011).

Larger differences were observed in MLF duration (Table 7, Figure 12). Generally, MLF took longer in Merlot 2 wines than in Merlot 1 wines. All MLFs finished with the exception of the spontaneous MLF of Sc wines in Merlot 2. The duration of the MLF inoculated with O. oeni PSU-1 in Merlot 1 was significantly lower in TdB and TdV compared to Sc wines. The same tendency was observed in Merlot 2 wines for the two *O. oeni* strains: TdB and TdV had a shorter MLF duration than Sc wines. This was not the case for *O. oeni* CH11 in Merlot 1, where the MLF took less time in Sc wine (8 days) than in TdB (13 days) or TdV (17 days) wines. This could be due to the strain specific interactions between these yeasts and LAB (Balmaseda et al., 2018).

The scenario found in Merlot 2 wines can be summarised as longer MLF with more difficulties involved in its development (Figure 12, Table 7). Wines from Merlot 1 and Merlot 2 resulted in similar ethanol concentration, pH and organic acid concentration (Table 8). Altogether, large differences were observed in the polyphenolic composition (Figure 13). Merlot 2 wines had a significantly higher concentration of anthocyanins, tannins and TPI, which have been previously related to harsh conditions for the development of MLF (Reguant et al., 2000). Their effect on LAB may be positive or negative depending on the nature and concentration of the compounds and on the bacterial strains (Breniaux et al., 2018; García-Ruiz et al., 2011). Recently, phenolic compounds have been described as stress compounds (Bech-Terkilsen et al., 2020). Indeed some of them, such as stilbenes, are related to an inhibition in malic acid degradation and CFU decline during MLF in *O. oeni* (Zimdars et al., 2021). The inhibition of polyphenols is dependent on their structure (Devi and Anu-Appaiah, 2018; García-Ruiz et al., 2011) and on the *O. oeni* strain (Zimdars et al., 2021).

A)

TPI	Sc	Sc-P	Sc-C	Sc-S	TdB	TdB-P	TdB-C	TdB-S	TdV	TdV-P	TdV-C	TdV-S
M1	30.2±4^{c}	33.1±0.8^{bc}	33.4±0.2^{abc}	31.9±0.2^{bc}	31.8±0.8^{bc}	33.7±0.3^{abc}	32.7±1.5^{bc}	32.6±0.3^{bc}	37.4±2^{a}	35.9±0.3^{ab}	35.4±0.1^{ab}	34.9±0.8^{ab}
M2	43.4±0.8^{bc}	41.6±0.9^{c}	41.6±0.5^{c}	41.7±0.5^{c}	46.7±1.8^{a}	45.4±1.3^{ab}	45.8±0.7^{ab}	45±0.4^{ab}	45±1.4^{ab}	44.1±0.8^{abc}	44±0.3^{abc}	43.3±0.4^{bc}

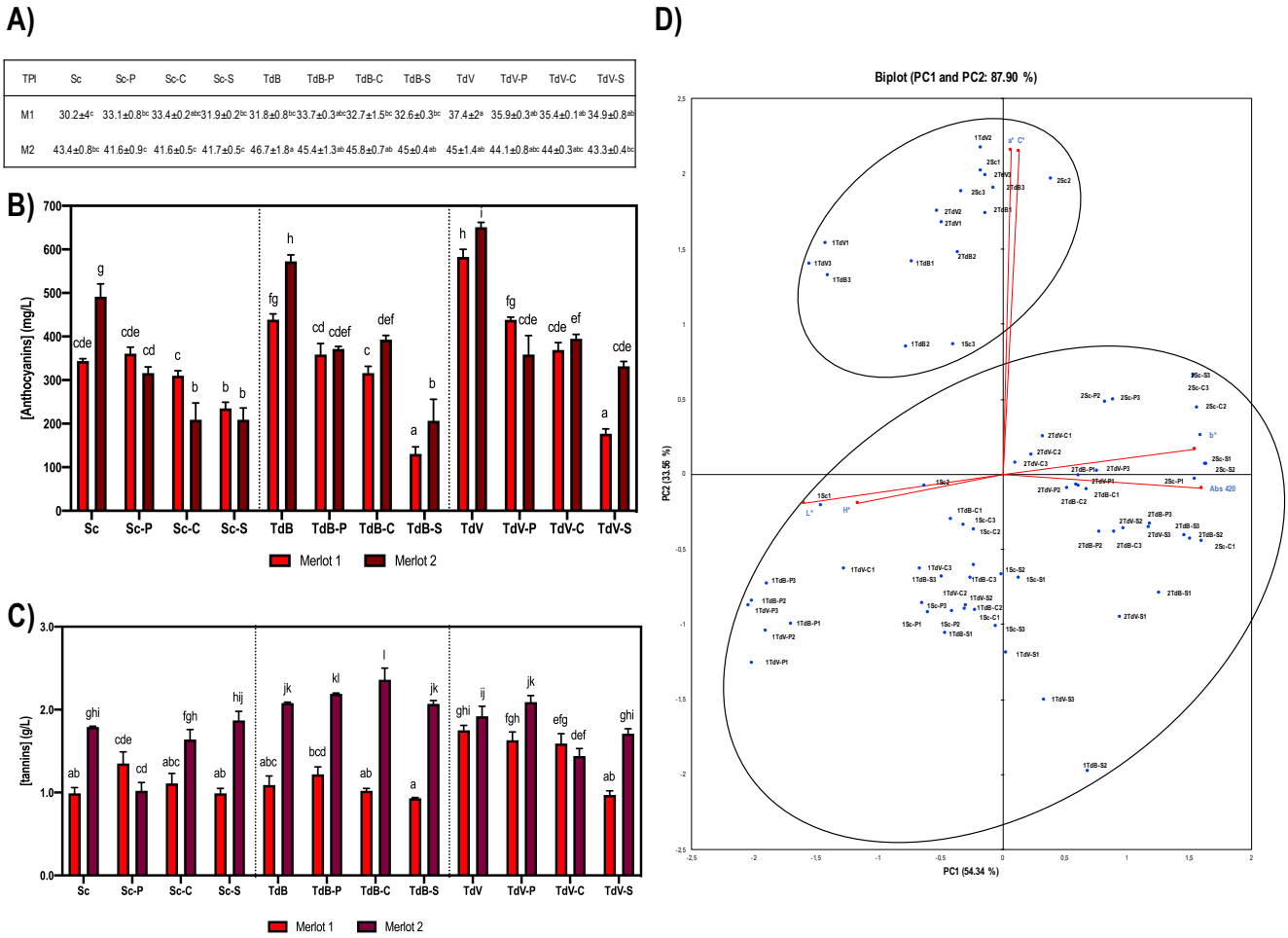

Figure 13. Polyphenolic and color parameters of wines after alcoholic and malolactic fermentation. M1 and M2 correspond to Merlot 1 and Merlot 2 grape musts. Sc, TdB and TdV correspond to the wines fermented with *S. cerevisiae*, *T. delbrueckii* Biodiva-*S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* respectively. P, C and S correspond malolactic fermentation strategy were *O. oeni* PSU-1, *O. oeni CH11* or non-*O. oeni* was inoculated. A) Total polyphenolic index (TPI). B) Anthocyanin concentration of wines. C) Tannin concentration of wines. All the values shown are the mean of triplicates ± SD. Statistics were calculated independently for each grape must. Values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison test. D) Principal component analysis (PCA) biplots of varimax rotated PCA for wine volatile composition in which observations (the three replicates of each wine) and variables are plotted. The last number of each observation indicates the replica number (1-3).

In these wines, the use of *T. delbrueckii* clearly resulted in shorter MLF in the inoculated wines and in successful spontaneous MLF, although high polyphenolic concentration was detected (Figure 13). The wine fermented just with *S. cerevisiae* in Merlot 2 could not undergo spontaneous MLF since the LAB viable population did not reach 10^2 CFU/mL in more than three months (data not shown). In general, calculated consumption rates were higher in *T. delbrueckii* wines (Table 7). In addition, high

consumption rates were observed in spontaneous MLF compared to inoculated wines because of a quick L-malic acid consumption in the final stages of MLF (Figure 13).

Analysis of the microbial population

The two grape musts had similar yeast concentrations: 9.6 x10^4 CFU/mL (total yeasts) and 1.7 x 10^4 CFU/mL (non-*Saccharomyces* yeasts) for Merlot 1 and 3.5 x 10^5 CFU/mL (total yeasts) and 6.1 x 10^4 CFU/mL (non-*Saccharomyces*) for Merlot 2. The inoculation of the selected yeasts was successful in both fermenting musts: the imposition of *T. delbrueckii* at 48h before *S. cerevisiae* inoculation, and the imposition of *S. cerevisiae* at the end of AF were in all cases 85% or higher (data not shown). In fact, by the end of AF, non-*Saccharomyces* were not detected. On the second day of fermentation, all wines, including those initially inoculated with *T. delbrueckii*, had a yeast population of around 10^8 CFU/mL (data not shown). After three days of fermentation, the viable population of non-*Saccharomyces* was lost (less than 10^2 CFU/mL) in all fermentations (data not shown), due to the high imposition ability of the inoculated *S. cerevisiae* strain, also observed in the previous vintage (**Chapter I: 1**).

No significant indigenous LAB population (<10 CFU/mL) was detected during the entire AF process (data not shown). This correlates with the previous data about spontaneous MLF where the LAB population needed more than a month to undergo the fermentation (Table 7). Generally, during the MLF the inoculated wines maintained a population of 1-4 x 10^7 CFU/mL until L-malic acid was completely consumed. In contrast, Sc wines of Merlot 2 lost 2 logarithmic units of viable *O. oeni* cells at the end of the MLF process (data not shown).

The imposition of the inoculated strain was successful and complete in most of the cases (Figure 14). *O. oeni* PSU-1 reached the total imposition (100%) in all wines. However, *O. oeni* CH11 was not completely imposed in TdB wines, especially in Merlot 1 where it represented just 20% of the analysed population. Indeed, wines inoculated with CH11 had the slowest MLFs (Table 7), even if it was imposed at the end of MLF. It is interesting to observe that these two *O. oeni* strains behaved differently, highlighting the strain-specific interaction between yeasts and *O. oeni* (Balmaseda et al., 2018). This could be related to a higher concentration of polyphenols

in Merlot 2, which allowed better adaptation of the oenological commercial strain CH11 upon the autochthonous microbiota.

Spontaneous MLFs were characterised by a strain diversity dependent on the grape maturity with the exception of the strain AiB9 that was detected in Merlot 1 TdV wine and Merlot 2 TdB wine (Figure 14). Different strain compositions characterised the obtained wines after spontaneous MLF (Figure 14). Nevertheless, some of them appeared in more than one wine (AiB5, AiB8, AiB9 and AiB13). Altogether, the previous fermenting yeasts affected the observed strain diversity at the end of MLF. Moreover, using *T. delbrueckii* somehow promoted the MLF performance since the spontaneous MLF of these wines was quicker than that of *S. cerevisiae* (Table 7).

The strain diversity observed in Sc and TdB wines was similar, but not their imposition percentages. In addition, TdB and TdV wines share these dominant strains. As a result, we observed a more different microbiota in TdV compared to Sc. Moreover, the suitability of *O. oeni* CH11 in TdB was low but more efficient in Merlot 2. Altogether, there is probably a higher concentration of inhibiting compounds, which could explain the non-successful spontaneous MLF in Sc wine and the low diversity observed at the end of MLF in TdB wine, which could be related to a higher concentration of some polyphenols (Figure 13).

General oenological parameters of wines

They were particularly homogenous (Table 8) in both wines Merlot 1 and 2. All wines after AF presented an L-malic acid concentration of around 1 g/L (data not shown). This little reduction from the must concentration (Suppl. Table S3) could be related to the yeast metabolism (Belda et al., 2015; du Plessis et al., 2017) and not to LAB activity since no L-lactic acid was detected in these wines (Table 8). D-lactic acid generally increased a little during the MLF performance, and it was only noticeable in the spontaneous MLFs with the exception of Sc Merlot 2 wine, which did not undergo MLF. *O. oeni* can assimilate citric acid as a source of energy in wines as a response to stress (Bartowsky and Henschke, 2004; Lonvaud-Funel, 1999), but its consumption is dependent on the *O. oeni* strain (Bartowsky and Henschke, 2004).

Table 7. Alcoholic (AF) and malolactic (MLF) fermentation duration and consumption rate of sugar and L-malic acid. Values shown are the mean of triplicates ± SD. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva and *S. cerevisiae*), TdV (*T. delbrueckii* Viniferm and *S. cerevisiae*) fermented wines. PSU, CH11 and Spontaneous refer to the MLF strategy where *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* were inoculated.

		Duration* (days)				Consumption rate+ (g/L·day)			
		AF	PSU	CH11	Spontaneous	AF	PSU	CH11	Spontaneous
Merlot 1	Sc	8	6 (14)	8 (16)	36 (44)	15.5 ± 1^{a}	0.60 ± 0.04^{aB}	0.34 ± 0.05^{aA}	0.18 ± 0.01^{aA}
	TdB	10	2 (12)	13 (23)	34 (44)	15.5 ± 0.5^{a}	1.77 ± 0.08^{aB}	0.22 ± 0.01^{aA}	0.18 ± 0.01^{aA}
	TdV	10	2 (12)	17 (23)	34 (44)	15.0 ± 2.1^{a}	1.86 ± 0.06^{aB}	0.14 ± 0.03^{aA}	0.14 ± 0.01^{aA}
Merlot 2	Sc	8	23 (31)	30 (38)	-	19.2 ± 0.6^{b}	0.03 ± 0.01^{aA}	0.39 ± 0.08^{aB}	-
	TdB	12	14 (26)	17 (29)	44 (56)	16.4 ± 0.9^{a}	0.16 ± 0.01^{bA}	0.48 ± 0.01^{bB}	0.40 ± 0.03^{bAB}
	TdV	12	17 (29)	21 (33)	40 (52)	17.1 ± 0.5^{a}	0.08 ± 0.01^{aA}	0.1 ± 0.01^{aB}	0.27 ± 0.04^{aAB}

***** Durations in brackets in MLF represent the length of the complete fermentative process (AF + MLF).

+ Calculation based on consumption rate of sugar as density (AF) and L-malic acid (MLF) considering the period of exponential decrease of these values. Statistics are calculated regarding to the values inside each square corresponding to each fermentation (AF or MLF) in the two Merlot grape musts.

$^{a–b}$. Lowercase indicate significant differences at $p \leq 0.05$ according to a Tukey post-hoc comparison test regarding to the yeast used. $^{A–B}$. Uppercase indicate significant differences at $p \leq 0.05$ regarding to the MLF strategy used.

Here, citric acid was consumed by *O. oeni* PSU-1 and the spontaneous MLF, but not by *O. oeni* CH11 in Merlot 1 wines (Table 8), and in general, its consumption in Merlot 2 wines was lower than in Merlot 1. As a result, *O. oeni* released acetic acid, increasing the volatile acidity, but as citric acid content was low, no remarkable changes in acetic acid concentration were observed.

An increase in the concentration of glycerol was detected in *T. delbrueckii* wines (Table 8). It can be explained because some non-Saccharomyces have a more active glyceropyruvic pathway than *S. cerevisiae* (Belda et al., 2015). The role of glycerol in *O. oeni* metabolism is still unclear (Balmaseda et al., 2018). In these vinifications little variation in glycerol concentration was observed after MLF compared to after AF. (Table 8).

Generally, wines fermented with *T. delbrueckii* resulted in higher pH levels (Table 8), which is usually associated with an improvement in MLF performance since it can attenuate the inhibitory effect of ethanol and medium chain fatty acids (MCFA). The variation in the pH, higher or lower, is very dependent on the medium where the yeast is fermenting, as seen in previous studies (**Chapter I: 1**; Martín-García et al., 2020). Only a small increase in pH was observed in wines after MLF (Table 8).

Mixed fermentations with *T. delbrueckii* are reported to decrease acetaldehyde content in wine (Benito, 2018). In the present work, the use of *T. delbrueckii* showed a tendency to decrease its concentration, which was significantly different in Merlot 2 wines (Table 8).

Succinic acid content in wine after AF was reduced in *T. delbrueckii* wines of Merlot 1 must (Table 8), being its concentration half in 1TdV wine compared to the control 1Sc wine. This reduction is interesting for the subsequent MLF since succinic acid acts as a competitive inhibitor of the malolactic enzyme, delaying or inhibiting the MLF (Balmaseda et al., 2018). In Merlot 2 wines no reduction was observed.

Sulphur dioxide was significantly reduced in *T. delbrueckii* wines (Table 8) as observed in the previous vintage (**Chapter I: 1**). Nevertheless, its concentrations detected were lower than the threshold of 35 mg/L, which is considered as inhibitory for some *O. oeni* strains (Lerm et al., 2010).

Although some non-*Saccharomyces* can reduce the ethanol content in wine (Balmaseda et al., 2018) and particularly *T. delbrueckii* (Benito, 2018), in our work no reduction in ethanol content was detected in either of the Merlot wines. Still, this ability is very dependent on the medium (**Chapter I: 1**).

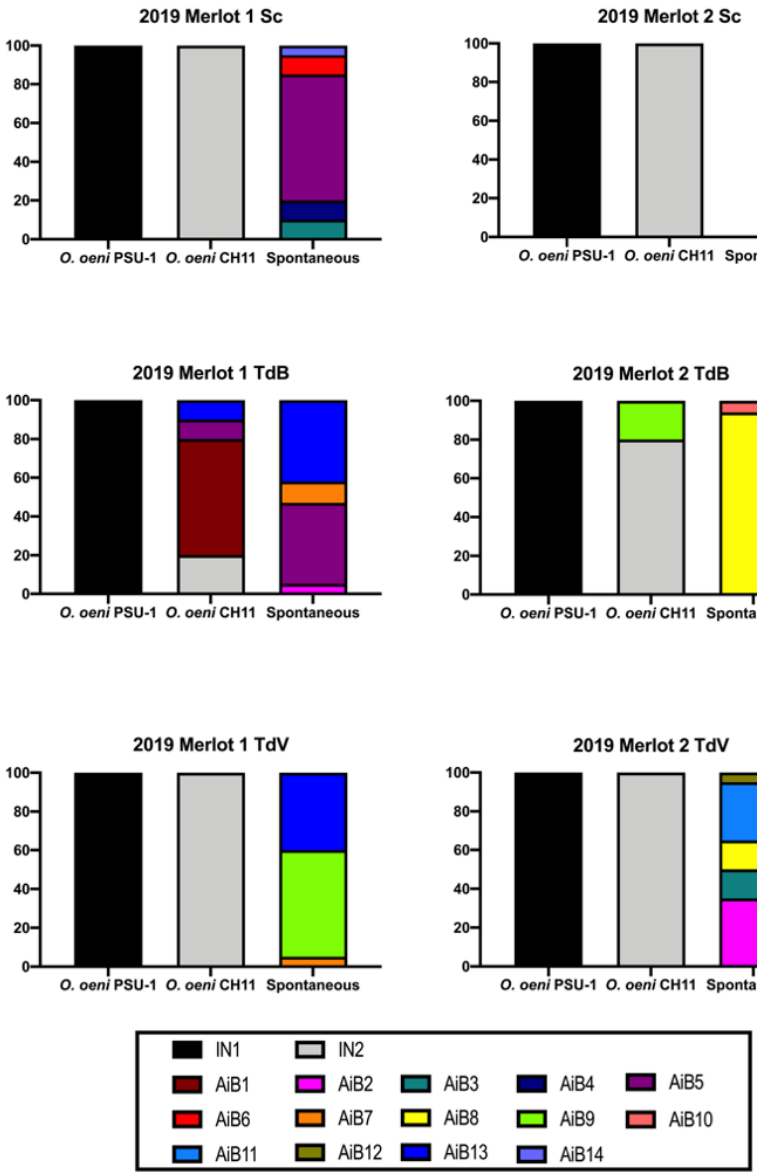

Figure 14. Percentage of imposition of the different VNTR profiles of *O. oeni* after malolactic fermentation. IN1 and IN2 refers to *O. oeni* PSU-1 and *O. oeni* CH11, respectively. AB named VNTR profiles correspond to naturally appeared profiles. Sc, TdB and TdV correspond to the wines fermented with *S. cerevisiae*, *T. delbrueckii* Biodiva-*S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* respectively. *O. oeni* PSU-1 and *O. oeni* CH11 correspond to the wines where one of each *O. oeni* strains was inoculated. Spontaneous refers to the wines where no *O. oeni* was inoculated.

Additionally, it should be considered that there may be other compounds, not determined in this work, that could explain the enhanced MLF performance in *T. delbrueckii* wines.

Analyses of volatile compounds

The wines produced had differences regarding the volatile composition (Figure 15A, Table 9). In general, wines fermented with *T. delbrueckii* and those with Merlot 2 grapes had a higher concentration of volatile compounds (Table 9).

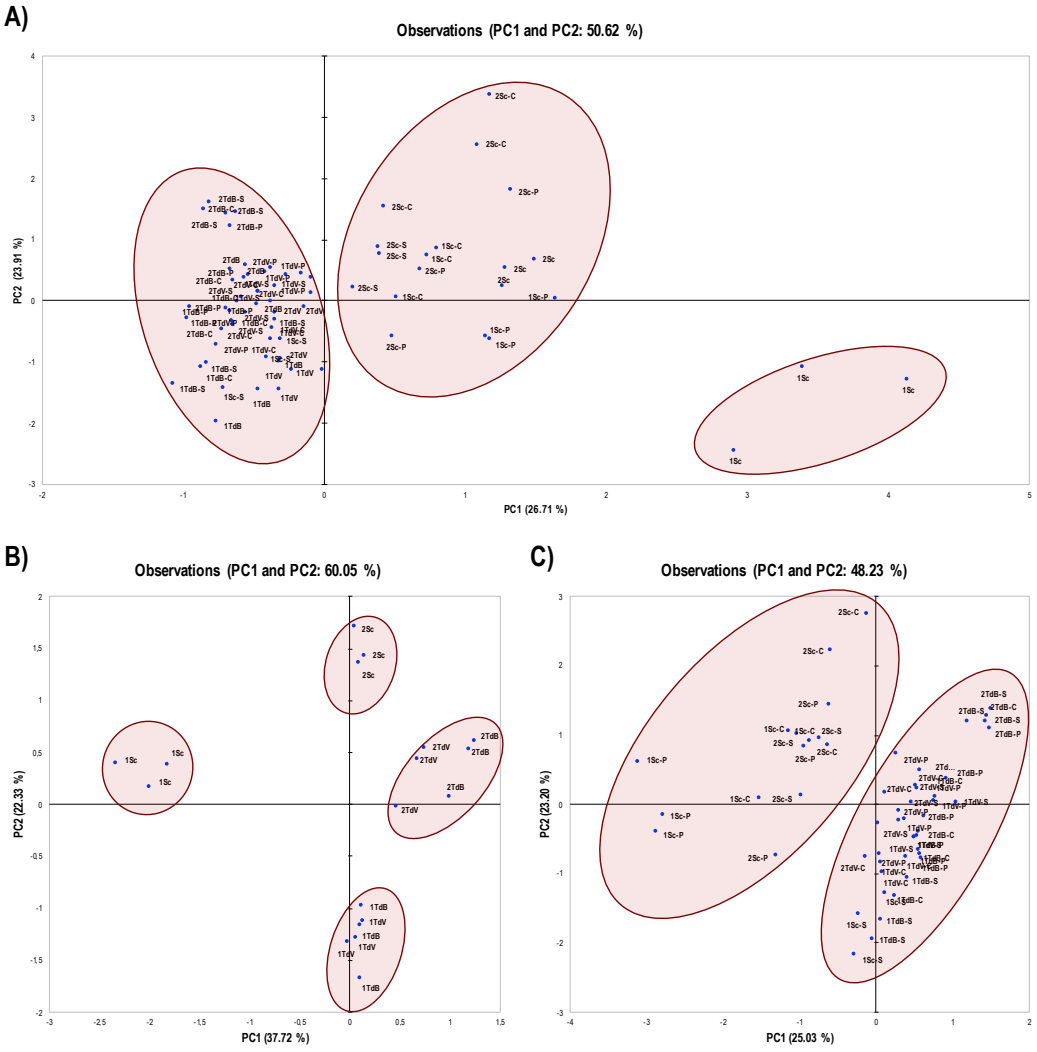

Figure 15. Principal component analysis (PCA) biplots of varimax rotated PCA for wine volatile composition in which observations are plotted. A) Produced wines with all strain combinations in the two grape musts. B) Wines after AF in the two grape musts. C) Wines after MLF in the two grape musts. 1 and 2 represent the two grape musts: Merlot1 and Merlot2 respectively. Sc, TdB and TdV correspond to the wines fermented with *S. cerevisiae*, *T. delbrueckii* Biodiva-*S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* respectively. P, C and S correspond malolactic fermentation strategy were *O. oeni* PSU-1, *O. oeni CH11* or non-*O. oeni* was inoculated..

Table 8. Oenological parameters of wines after alcoholic and malolactic fermentations. Values shown are the means of triplicates ± SD. Statistics were calculated independently for each grape variety. Sc, TdB and TdV correspond to *S. cerevisiae*, *T. delbrueckii* Biodiva- *S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated. n.d.: not detected.

	L-lactic acid (g/L)	D-lactic acid (g/L)	Acetic acid (g/L)	Citric acid (g/L)	Glycerol (g/L)	pH	Acetaldehyde (mg/L)	Succinic acid (g/L)	SO_2 T (mg/L)	Ethanol (% vol/vol)
Merlot 1										
Sc	n.d.^{d}	0.17 ± 0.01^{efg}	0.19 ± 0.01^{e}	0.13 ± 0.01^{ab}	6.1 ± 0^{c}	3.3 ± 0^{cd}	45.7 ± 1.53^{a}	0.24 ± 0.02^{a}	17 ± 1^{a}	13.1 ± 0.2^{a}
Sc-P	1.50 ± 0.01^{ab}	0.18 ± 0.01^{defg}	0.29 ± 0.02^{ab}	0.03 ± 0.01^{c}	5.8 ± 0.2^{c}	3.4 ± 0^{b}				
Sc-C	1.50 ± 0.02^{bc}	0.18 ± 0.01^{defg}	0.25 ± 0.01^{cd}	0.12 ± 0.01^{ab}	6 ± 0.3^{c}	3.4 ± 0^{bc}				
Sc-S	1.50 ± 0.6^{bc}	0.19 ± 0.02^{cdef}	0.31 ± 0.02^{a}	n.d.^{cd}	6.2 ± 0.1^{c}	3.2 ± 0.1^{d}				
TdB	n.d.^{d}	0.15 ± 0.01^{g}	0.19 ± 0.03^{e}	0.15 ± 0.02^{a}	7.4 ± 0.2^{ab}	3.4 ± 0^{bc}	42.8 ± 0.71^{a}	0.19 ± 0.01^{b}	11.7 ± 1.5^{b}	13 ± 0.2^{a}
TdB-P	1.55 ± 0.04^{ab}	0.21 ± 0.01^{cd}	0.29 ± 0.02^{ab}	n.d.^{d}	7 ± 0.2^{ab}	3.4 ± 0.1^{ab}				
TdB-C	1.48 ± 0.05^{c}	0.16 ± 0.02^{fg}	0.22 ± 0.01^{cde}	0.12 ± 0.02^{ab}	6.9 ± 0.1^{b}	3.4 ± 0^{ab}				
TdB-S	1.46 ± 0.01^{bc}	0.26 ± 0.01^{a}	0.31 ± 0.02^{ab}	n.d.^{d}	7.1 ± 0.1^{ab}	3.4 ± 0^{ab}				
TdV	n.d.^{d}	0.20 ± 0.01^{cde}	0.19 ± 0.02^{e}	0.13 ± 0.01^{ab}	7.3 ± 0.2^{ab}	3.4 ± 0^{bc}	41.5 ± 1.6^{a}	0.12 ± 0.02^{c}	13.3 ± 2.1^{ab}	13.1 ± 0.2^{a}
TdV-P	1.53 ± 0.02^{a}	0.25 ± 0.01^{ab}	0.27 ± 0^{bc}	n.d.^{d}	7.6 ± 0.3^{a}	3.5 ± 0^{a}				
TdV-C	1.40 ± 0.05^{c}	0.22 ± 0.02^{bc}	0.22 ± 0.01^{de}	0.11 ± 0.01^{b}	7.5 ± 0.3^{a}	3.5 ± 0^{a}				
TdV-S	1.48 ± 0.04^{bc}	0.27 ± 0.01^{a}	0.29 ± 0.01^{ab}	n.d.^{d}	7.3 ± 0.2^{ab}	3.2 ± 0^{d}				

Merlot 2										
Sc	n.d.^{d}	0.16 ± 0.02^{c}	0.22 ± 0.02^{ef}	0.16 ± 0.01^{ab}	6.5 ± 0.2^{bc}	3.3 ± 0.1^{a}	52.2 ± 4.9^{a}	0.27 ± 0.01^{a}	18 ± 2^{a}	13.3 ± 0.2^{a}
Sc-P	1.40 ± 0.01^{c}	0.16 ± 0.01^{bc}	0.22 ± 0.01^{cd}	0.12 ± 0.02^{bc}	6.9 ± 0.1^{c}	3.4 ± 0^{a}				
Sc-C	1.19 ± 0.05^{bc}	0.19 ± 0.01^{bc}	0.28 ± 0.01^{de}	0.13 ± 0.01^{ab}	6.3 ± 0.1^{bc}	3.4 ± 0^{a}				
Sc-S	0.10 ± 0.05^{d}	0.17 ± 0.02^{bc}	0.22 ± 0.01^{ef}	0.16 ± 0.01^{ab}	6.3 ± 0.4^{c}	3.2 ± 0^{ab}				
TdB	n.d.^{d}	0.15 ± 0.02^{c}	0.19 ± 0.01^{f}	0.14 ± 0.01^{abc}	7.4 ± 0.3^{a}	3.3 ± 0^{a}	42.8 ± 0.7^{b}	0.27 ± 0.04^{a}	13.5 ± 0.5^{b}	13 ± 0.7^{a}
TdB-P	1.51 ± 0.04^{ab}	0.17 ± 0.02^{bc}	0.27 ± 0.01^{cd}	0.13 ± 0.02^{bc}	7.2 ± 0.1^{a}	3.4 ± 0.1^{a}				
TdB-C	1.40 ± 0.12^{ab}	0.17 ± 0.02^{bc}	0.29 ± 0.02^{bcd}	0.14 ± 0.02^{ab}	7.6 ± 0.2^{a}	3.4 ± 0.1^{a}				
TdB-S	1.55 ± 0.03^{a}	0.27 ± 0.01^{a}	0.29 ± 0.01^{bcd}	0.07 ± 0^{d}	7.1 ± 0.2^{ab}	3.3 ± 0^{a}				
TdV	n.d.^{d}	0.18 ± 0.03^{bc}	0.27 ± 0.01^{cd}	0.15 ± 0.01^{ab}	7.5 ± 0.4^{a}	3 ± 0.2^{b}	43.1 ± 3.12^{b}	0.29 ± 0.02^{a}	14.3 ± 1.2^{b}	13.4 ± 0^{a}
TdV-P	1.38 ± 0.12^{ab}	0.22 ± 0.05^{ab}	0.31 ± 0.03^{bc}	0.07 ± 0.01^{d}	7.7 ± 0.2^{a}	3.4 ± 0^{a}				
TdV-C	1.29 ± 0.04^{ab}	0.22 ± 0.01^{ab}	0.36 ± 0.01^{a}	0.17 ± 0.02^{a}	7.4 ± 0.2^{a}	3.3 ± 0.2				
TdV-S	1.55 ± 0.03^{ab}	0.27 ± 0.01^{a}	0.32 ± 0.01^{ab}	0.10 ± 0.03^{cd}	7.2 ± 0^{a}	3.4 ± 0^{a}				

$^{a–g}$. Values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison test.

As a result of PCA (Figure 15A), wines were separated through the PC2 into those fermented only with *S. cerevisiae* and those sequentially fermented with both yeasts, with the exception of Sc-S wines. Almost all the volatile components of the PCA point to TdB and TdV (Suppl. Figure S5), which were the wines with the highest concentration of volatile compounds (Table 9, Suppl. Table S4).

Table 9. Concentrations of wine volatile compounds (mg/L) after AF grouped as family compounds. SCFA (propionic, isobutyric, butyric 3-methylbutanoic and valeric acids), MCFA (hexanoic, octanoic and decanoic acids), Ethyl esters of FA (ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate), Fusel alcohols (2-methyl-propanol, 1-butanol, 2- methyl-1-butanol, benzyl alcohol, 2-phenylethanol, 1-propanol, 1-pentanol, 1-hexanol and cis-3-hexen-1-ol), Fusel alcohol acetates (2-phenylethanol acetate, isobutyl acetate and isoamyl acetate). Sc, TdB and TdBV correspond to *S. cerevisiae*, *T. delbrueckii* Biodiva- *S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* fermented wines, respectively. Values shown are the mean of triplicates ± SD. Statistics were calculated independently for each grape must.

	Σ SCFA (mg/L)	Σ MCFA (mg/L)	Σ Ethyl esters of FA (mg/L)	Ethyl lactate (mg/L)	Σ Fusel alcohols (mg/L)	Σ Fusel alcohol acetates (mg/L)
Merlot 1						
Sc	6.7 ± 1.1^{a}	3.6 ± 0.4^{b}	6.3 ± 0.3^{c}	7.7 ± 0.5^{a}	245.9 ± 28.8^{a}	5.7 ± 0.3^{b}
TdB	9.1 ± 1.2^{ab}	0.5 ± 0.1^{a}	4.0 ± 0.5^{b}	6.2 ± 0.2^{a}	220.0 ± 24.8^{a}	3.1 ± 0.2^{a}
TdV	9.5 ± 0.5^{b}	0.6 ± 0.1^{a}	2.9 ± 0.1^{a}	6.0 ± 0.6^{a}	221.2 ± 4.9^{a}	3.3 ± 0.2^{a}
Merlot 2						
Sc	17.0 ± 1.1^{b}	0.8 ± 0.0^{b}	6.1 ± 0.4^{a}	0 ± 0^{a}	228.0 ± 5.9^{a}	5.5 ± 0.1^{b}
TdB	13.6 ± 2.0^{ab}	0.5 ± 0.1^{a}	6.0 ± 0.7^{a}	7.4 ± 0.1^{b}	249.8 ± 7.6^{a}	5.3 ± 0.1^{ab}
TdV	11.5 ± 2.4^{a}	0.5 ± 0.0^{a}	6.6 ± 0.2^{a}	8.6 ± 0.3^{c}	235.3 ± 12.6^{a}	5.1 ± 0.1^{a}

* $^{a–c}$. values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison test. n.d.: not detected

In contrast 1Sc wines are plotted on the opposite side, where there are mainly ethyl esters and MCFA. In this case, *S. cerevisiae* wines (1Sc and 2Sc) showed a poor volatile composition related to their less aromatic enzymatic activities (Romano et al., 2003) and lower maturation level of the grapes (Ferreira and Lopez, 2019). However, the maturity level did not have a significant impact when *T. delbrueckii* fermented. TdB and TdV wines from Merlot 1 and Merlot 2 were clustered in the most aromatic group (Figure 15A).

When we analysed wines after AF (Figure 15B), we observed large differences. Samples were clustered in four groups: *T. delbrueckii* wines from Merlot 1, the same

from Merlot 2 and two clusters for *S. cerevisiae* wines. It is remarkable that although TdB and TdV showed high homogeneity in the two grape musts, Sc wines had much more variability. 1Sc and 2Sc wines had significantly higher concentrations of MCFA (Table 9), more remarkable in 1Sc. Previous studies showed that the use of non-*Saccharomyces* can reduce the concentration of MCFA (Balmaseda et al., 2018), which can be toxic to *O. oeni* (Capucho and San Romao, 1994), by destabilizing its cell membrane (Carreté et al., 2002). Nevertheless, the concentration of this compound family was low, then it should not have a large impact on *O. oeni*. The contribution to SCFA was mainly due to high isobutyric and propionic acid concentrations (Suppl. Table S4). In this sense, higher concentrations of isobutyric acid have been recently associated with *T. delbrueckii* (Sereni et al., 2020). Sc wines had the highest ethyl ester of FA concentration in Merlot 1 (Table 9). Low concentrations of ethyl lactate were quantified in wines after AF (Table 9), which increased at the end of the MLF due to the LAB metabolism (Suppl. Table S4), as previously known (Liu, 2002).

Regarding volatile composition after MLF (Figure 15C), two clusters of wines can be observed. PC1 separates Merlot 1 and Merlot 2 wines and PC2 separates Sc wines from TdB and TdV wines, with some exceptions. The most aromatic wines are grouped in the positive PC1 and PC2 quadrant: 2TdB and 2TdV wines. As described, the use of different non-*Saccharomyces* enhances the wine volatile composition (Englezos et al., 2016a; Liu et al., 2016; Tofalo et al., 2016; Tufariello et al., 2020). Of course, this was clearer in Merlot 2 wines, which came from more mature grapes.

Colour parameters and phenolic characterization

The different maturity levels of Merlot 1 and Merlot 2 grapes resulted in higher TPI, anthocyanin and tannin concentrations in Merlot 2 wines (Figure 13).

TPI varied from 30 to 37 in Merlot 1 and from 32 to 47 in Merlot 2 wines (Figure 13A). The use of *T. delbrueckii* could be noted in wines after AF, and this tendency was clearer in Merlot 2 for which the two *T. delbrueckii* wines had a significantly higher index than the *S. cerevisiae* wine. The higher levels of polyphenols in non-*Saccharomyces* wines have been previously described (Escribano-Viana et al., 2019).

The anthocyanin concentration was also greater after AF as a consequence of the use of *T. delbrueckii* (Figure 13B), and particularly in the case of the *T. delbrueckii* Viniferm strain in Merlot 2 wine. This behaviour seems to be strain specific (Carew et al., 2013; Chen et al., 2018); therefore, the selection of strains in terms of anthocyanin adsorption is crucial for red winemaking (Benito, 2018a). After MLF, we found that its decreased in all wines (Costello et al., 2012; Davis et al., 1985), and a dramatic drop in concentration was observed in spontaneous MLF, probably because it took a long time to finish (Figure 13, Table 7).

Tannin concentration varied from 1 to 2.4 g/L in all wines (Figure 13C). It was also significantly higher in *T. delbrueckii* wines (Figure 13C), especially in Merlot 2. Indeed, wines coming from Merlot 2 grapes can be considered as high tannic wines as they have more tannins than the average for this variety (Harbertson et al., 2008). *T. delbrueckii* is described as a non-*Saccharomyces* with low adsorption of polyphenols (Benito, 2018a).

According to the colour parameters, wines were clustered in two groups in the PCA (Figure 13D). One of them, characterised by higher a* and Chroma values, grouped AF wines except for some replicates of Sc wines. Wines after MLF were grouped in another cluster in the opposite direction, with higher heterogeneity. In addition, wines in the two clusters tended to be closer to other wines produced with the same Merlot maturity level.

In summary, the use of *T. delbrueckii* increased the TPI due to the accumulation of both anthocyanin and tannins, mainly after AF. After MLF there was a tendency for the polyphenolic composition to decrease, probably due to oxidation or precipitation or even attachment to yeast cell walls (Escribano-Viana et al., 2019), or also due to the interaction with *O. oeni* cell envelopes (Campos et al., 2003, 2009). A dramatic fall was observed in anthocyanin concentrations after spontaneous MLF, presumably as a consequence of a long fermenting period.

Table 10. Results of the sensorial analysis from both, triangular and classification tests. 1 and 2 refers to Merlot 1 or Merlot 2 grapes. Sc, TdB and TdV correspond to *S. cerevisiae*, *T. delbrueckii* Biodiva- *S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.

Triangular tests			
		Preference*	
Compared wines	Positive identifications	A	B
1Sc vs 1TdB	9/25	7 ^{NS}	2
1TdB vs 1TdV	15/25	3	12 $^{\alpha}$
1Sc vs 1TdV	15/25	11 $^{\alpha}$	4
1Sc-P vs 1Sc-C	4/17	3 ^{NS}	1
1Sc-C vs 1Sc-S	10/17	10 ^{ß}	0
1Sc-P vs 1Sc-S	12/17	9	3 $^{\alpha}$
2ScC vs 2TdB-C	8/13	1	7 $^{\alpha}$
2Sc-C vs 2TdV-C	8/13	0	8 ^{ß}
2TdB-C vs 2TdV-C	5/13	5 $^{\alpha}$	0
2Sc-P vs 2TdB-P	8/13	2	6 ^{NS}
2Sc-P vs 2TdV-P	8/13	3	5 ^{NS}
2TdB-P vs 2TdV-P	9/13	4	5 ^{NS}
Classification test			
Attribute	Wines classification as increasing intensity of the attribute§		
Red fruit	$1Sc^{1,2,3}$ > $2Sc^{4}$ > 1Sc-S > 2Sc-P > 2TdB-S > 2TdB-P^{1} > 1Sc-P^{2} > 1TdB-$P^{3,4}$		
Lactic character	$2Sc^{1}$ > $1Sc^{2}$ > 2TdB-S > 1Sc-P > 2TdB-P > 2Sc-P > 1Sc-S > 1TdB-$P^{1,2}$		
Astringency	1Sc-$S^{1,2,3}$ > 1Sc-$P^{4,5}$ > 1Sc > 2TdB-S > 1TdB-P > 2Sc-P^{2} > 2TdB-$P^{3,4}$ > $2Sc^{1,5}$		

* Only the preference of positive identifications is shown. A and B correspond to the first and second wine for each comparison. ^{NS}, No significant; $^{\alpha}$, Significant at $p < 0.05$; ^{ß}, Significant at $p < 0.001$.

It is important to highlight that a higher concentration of phenolic compounds is usually related to more stressful conditions for MLF (Bech-Terkilsen et al., 2020). Even so, wines fermented with *T. delbrueckii*, with a higher concentration of these compounds (Figure 13), were also the ones where MLF performance was enhanced, especially with strain Biodiva (Table 7, Figure 13). Therefore, the use of *T. delbrueckii*

seems to promote changes in composition that favour *O. oeni* adaptation to wine stressful conditions, as seen in **Chapter I: 1**.

Wine tasting

The results of the triangle tests, including those after AF and MLF, are shown in Table 10. Regarding wines after AF tasters preferred, in general, wines only fermented with *S. cerevisiae*, although they were not able to distinguish 1Sc and 1TdB. Interestingly, wines produced with the two strains of *T. delbrueckii* resulted in significantly different wines, and the TdV wine was the preferred one. The results were robust for the wines after MLF. Tasters clearly preferred the inoculated ones with *O. oeni* from those spontaneously fermented, and no differences were observed comparing strains PSU-1 and CH11. In addition, tasters preferred *T. delbrueckii* wines after MLF and differentiated them from *S. cerevisiae* ones.

Considering the information obtained from the triangle tests, eight representatives wines were selected to perform a classification test according to three attributes (Table 10, below). Wines fermented only with *S. cerevisiae* were the ones with less intensity of the red fruit aroma attribute. However, after MLF these wines showed an increased red fruit aroma. This is also related to the volatile composition of wines, and *S. cerevisiae* wines were the least aromatic of the produced wines (Figure 15). The lactic character typical of wines after MLF was correctly assessed because wines after MLF showed an increased intensity in this attribute. Nevertheless, not many significant differences were found. Considering astringency, wines from Merlot 2 grapes, in general, had higher astringency than Merlot 1 wines. This correlates with the higher TPI and tannin concentrations observed in Merlot 2 wines (Figure 13).

Conclusion

The use of *T. delbrueckii* in more mature grape wines reduced the duration of the fermentative process and enabled spontaneous MLF. In this way, the diversity of *O. oeni* strains was dependent on the maturity level and the fermenting yeast combination. Volatile complexity and polyphenolic composition was enhanced due to the use of *T. delbrueckii*, mainly in wines made from more mature grapes. These effects

were more remarkable for some strains, such as Biodiva for promoting MLF or Viniferm for the polyphenolic concentration. Therefore, careful attention should be given to strain selection and yeast-*O. oeni* strain compatibility to benefit from their oenological advantages in red winemaking.

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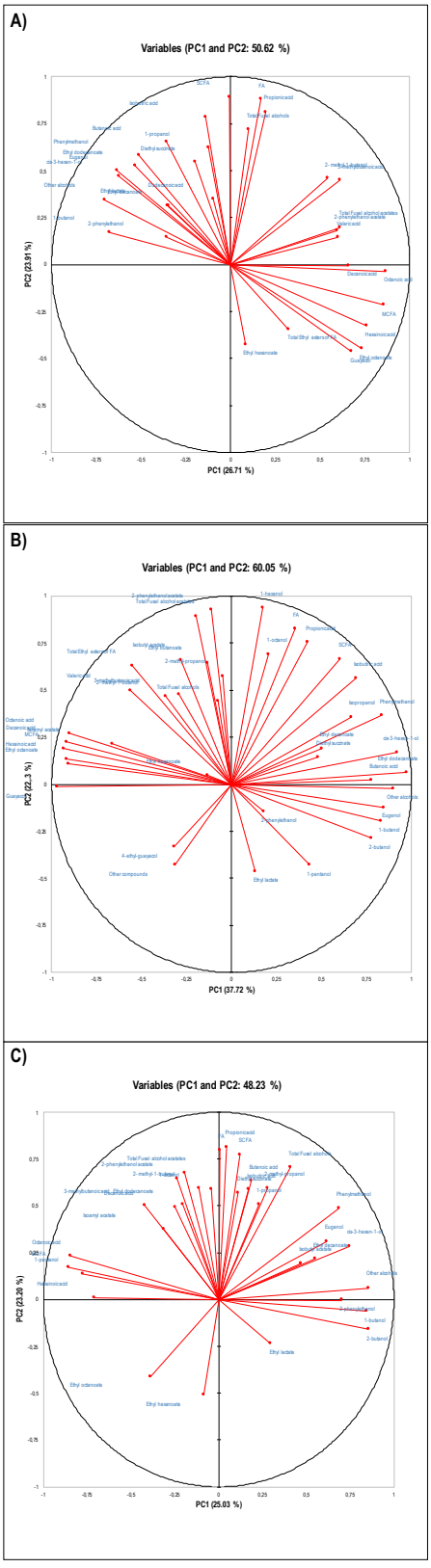
Supplementary Figures

Suppl. Figure S5. Principal component analysis (PCA) biplots of varimax rotated PCA for wine volatile composition in which variables are plotted. A) Produced wines with all strain combinations in the two grape musts. B) Wines after AF in the two grape musts. C) Wines after MLF in the two grape musts.



Supplementary Tables

Suppl. Table S3. Oenological parameters of the two grape musts (Merlot 1 and Merlot 2) before fermentation.

	Density (g/L)	pH	Total acidity (g tartaric acid/L)	L-malic acid (g/L)	Citric acid (g/L)	Acetic acid (g/L)	NOPA (mg/L)	NH_4 (mg/L)
Merlot 1	1087.8	3.22	5.66	1.22	0.35	0.03	75	25
Merlot 2	1090.8	3.26	4.72	1.19	0.39	0.04	86	33

Suppl. Table S4. Concentrations of wine volatile compounds (mg/L). SCFA (propionic, isobutyric, butyric 3-methylbutanoic and valeric acids), MCFA (hexanoic, octanoic and decanoic acids), Ethyl esters of FA (ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate), Fusel alcohols (2-methyl-propanol, 1-butanol, 2- methyl-1-butanol, benzyl alcohol, 2-phenylethanol, 1-propanol, 1-pentanol, 1-hexanol and cis-3-hexen-1-ol), Fusel alcohol acetates (2-phenylethanol acetate, isobutyl acetate and isoamyl acetate). Sc, TdB and TdBV correspond to S. cerevisiae, T. delbrueckii Biodiva- S. cerevisiae and T. delbrueckii Viniferm-S. cerevisiae fermented wines, respectively. Values shown are the mean of triplicates ± SD.

	Sc	Sc-P	Sc-C	Sc-S	TdB	TdB-P	TdB-C	TdB-S	TdV	TdV-P	TdV-C	TdV-S
Merlot 1												
Propionic acid	2.2 ± 0.4	3 ± 0.3	4 ± 0	2.1 ± 0.1	2.1 ± 0.1	3.4 ± 0.4	2.5 ± 0.2	2.5 ± 0.4	2.5 ± 0	2.9 ± 0.2	2.4 ± 0.2	2.9 ± 0
Isobutiric acid	3 ± 0.6	6.5 ± 0	6.3 ± 0.2	8.8 ± 0.1	5.4 ± 1	8.4 ± 0.9	6.1 ± 1	6.5 ± 0.3	5.4 ± 0.4	7.3 ± 0.4	5.9 ± 0.2	6.9 ± 0
Butiric acid	n.d.	0.3 ± 0	0.6 ± 0.1	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0	0.3 ± 0	0.5 ± 0
3-methylbutanoic acid	1.2 ± 0.3	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0	1.1 ± 0.1	1 ± 0.1	1.1 ± 0	1.2 ± 0.1	1.1 ± 0	1.2 ± 0.1
Pentanoic acid	0.3 ± 0.1	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0	0.3 ± 0.1	0.2 ± 0	0.3 ± 0.1	0.3 ± 0	0.3 ± 0	0.3 ± 0.1
Hexanoic acid	1.9 ± 0.3	1.5 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Octanoic acid	1.2 ± 0.3	1 ± 0	1 ± 0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0	0.5 ± 0	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0	0.6 ± 0	0.6 ± 0
Decanoic acid	0.5 ± 0.1	0.2 ± 0	0.2 ± 0.1	n.d.	n.d.	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	n.d.
Dodecanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0	n.d.	0.1 ± 0	n.d.	n.d.

SCFA	6.7 ± 1.1	11.2 ± 0.4	12.5 ± 0.5	12.5 ± 0.3	9.1 ± 1.2	13.4 ± 1.2	10.2 ± 1.2	10.5 ± 0.7	9.5 ± 0.5	11.9 ± 0.6	11.0 ± 0.5	11.9 ± 0.2
MCFA	3.6 ± 0.4	2.6 ± 0.1	1.2 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0	0.6 ± 0	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0	0.7 ± 0	0.6 ± 0.1
FA	10.2 ± 0.9	13.8 ± 0.5	13.7 ± 0.4	13 ± 0.4	9.6 ± 1.2	14 ± 1.3	10.8 ± 1.2	11.2 ± 0.8	1n.d..5	12.7 ± 0.6	10.7 ± 0.5	12.5 ± 0.1
Ethyl hexanoate	3.1 ± 0.3	1.9 ± 0.1	1.7 ± 0.4	2.5 ± 0.4	2.5 ± 0.4	3.1 ± 0.2	3.1 ± 0.2	2.9 ± 0.8	1.5 ± 0.1	2.4 ± 0.2	3.4 ± 0.3	n.d.
Ethyl octanoate	4 ± 0.5	1.3 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0	1 ± 0.1	1 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1 ± 0.2
Ethyl butanoate	2.2 ± 0.2	2 ± 0.2	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0.2 ± 0.1	0.1 ± 0	2.3 ± 0
Ethyl lactate	6.3 ± 0.3	86.5 ± 3.4	102.7 ± 5.3	120.5 ± 3.8	6.2 ± 0.2	126.3 ± 11.7	96.4 ± 3.2	127.5 ± 8.9	6 ± 0.6	128.4 ± 1.6	115.7 ± 2.3	157.3 ± 12.6
Ethyl decanoate	n.d.	n.d.	n.d.	n.d.	n.d.	0.3 ± 0.2	0.4 ± 0.1	n.d.	n.d.	n.d.	0.3 ± 0.1	n.d.
Diethyl succinate	n.d.	0.1 ± 0	0.2 ± 0	n.d.	n.d.	n.d.	0.2 ± 0.1	n.d.	n.d.	n.d.	0.1 ± 0	0.2 ± 0
Ethyl dodecanoate	n.d.	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0.1	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0
Ethyl esters of FA	9.3 ± 0.6	5.6 ± 0.3	3 ± 0.4	4 ± 0.5	4 ± 0.5	5 ± 0.2	4.9 ± 0.2	4.3 ± 1	2.9 ± 0.1	3.7 ± 0.4	5.2 ± 0.3	3.5 ± 0.2
2-methyl-propanol	64.1 ± 3.8	54.7 ± 0.7	66.3 ± 1	56.4 ± 9.7	56.4 ± 9.7	57.1 ± 1.6	56.2 ± 9.1	43.5 ± 2	53.8 ± 2.3	60.4 ± 1.3	56.5 ± 1.1	64.6 ± 8
1-butanol	0.8 ± 0	0.8 ± 0.2	0.8 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	1.4 ± 0	1.4 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.5 ± 0.2
2- methyl-1-butanol	116.3 ± 21.6	106.2 ± 7.2	109.8 ± 5.5	94.5 ± 4.3	94.5 ± 4.3	100.2 ± 2	102.2 ± 9.1	89.3 ± 10.3	103.4 ± 1.4	115.8 ± 0.9	107.6 ± 2.2	112.5 ± 2.5
Phenylmethanol	1.6 ± 0.3	1.9 ± 0.1	2.6 ± 0.4	2.5 ± 0.3	2.5 ± 0.3	3 ± 0.1	2.9 ± 0.4	2.8 ± 0.3	2.2 ± 0.1	3.1 ± 0.2	2.7 ± 0	3.1 ± 0
2-phenylethanol	61.6 ± 7.8	55.1 ± 4	56.9 ± 0.8	64.1 ± 12.7	64.1 ± 12.7	64.8 ± 3.3	67.4 ± 3.5	60.4 ± 8.4	59.2 ± 4.7	74.7 ± 1.3	69.4 ± 0.7	68.6 ± 0.6
1-propanol	n.d.	n.d.	15.4 ± 3.7	n.d.	n.d.	16.2 ± 1.2	15.8 ± 4.2	13.2 ± 2.1	n.d.	13.6 ± 0.7	12 ± 1	3.2 ± 0
1-pentanol	n.d.	0.8 ± 0.1	0.4 ± 0.1	0.1 ± 0	0.1 ± 0	n.d.	0.1 ± 0	n.d.	0.2 ± 0	n.d.	n.d.	0.1 ± 0.1
1-hexanol	1.5 ± 0.3	1.2 ± 0.1	1.2 ± 0	1 ± 0.1	1 ± 0.1	4.7 ± 2.8	0.8 ± 0	0.7 ± 0.1	0.9 ± 0	1 ± 0	0.9 ± 0	0.8 ± 0
cis-3-hexen-1-ol	n.d.	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0	0.3 ± 0.1	0.2 ± 0	0.2 ± 0
Fusel alcohols	245.9 ± 28.8	220.8 ± 11.1	253.5 ± 10.5	220 ± 24.8	220 ± 24.8	247.8 ± 1.4	247.1 ± 26.3	211.5 ± 20.5	221.2 ± 4.9	270.2 ± 0.3	250.5 ± 4.1	254.6 ± 11.4
2-phenylethanol acetate	4.8 ± 0.1	3.9 ± 0.3	3.5 ± 0.4	2.8 ± 0.1	2.8 ± 0.1	3.3 ± 0.1	3.1 ± 0.3	2.5 ± 0.4	3 ± 0.2	3.5 ± 0	2.9 ± 0.1	2.9 ± 0
Isobutil acetate	0.4 ± 0.2	n.d.	0.2 ± 0.2	n.d.	n.d.	0.2 ± 0.1	0.2 ± 0	0.2 ± 0.1	n.d.	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0
Isoamil acetate	0.5 ± 0.1	0.5 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0
Fusel alcohols acetates	5.7 ± 0.3	4.5 ± 0.5	4 ± 0.6	3.1 ± 0.2	3.1 ± 0.2	3.8 ± 0	3.6 ± 0.5	2.9 ± 0.5	3.3 ± 0.2	4.2 ± 0.1	3.6 ± 0.1	3.5 ± 0
Isopropanol	0.2 ± 0	n.d.	1 ± 0.3	0.2 ± 0	0.2 ± 0	0.7 ± 0.2	0.2 ± 0.4	n.d.	0.2 ± 0.1	0.8 ± 0.4	0.5 ± 0	1.1 ± 0

2-butanol	2.3 ± 0.4	2.5 ± 0.2	2.8 ± 0.6	4.5 ± 0	4.5 ± 0	5.2 ± 0	5.5 ± 1.2	4.3 ± 0.5	4.2 ± 0.3	5.4 ± 0.2	5 ± 0.5	5.2 ± 0
1-octanol	n.d.	0.8 ± 0.1	1.1 ± 0.3	n.d.	n.d.	0.6 ± 0	1.4 ± 0.1	1 ± 0.2	n.d.	1.5 ± 0.1	0.6 ± 0.2	0.6 ± 0
Merlot 2												
Propionic acid	5.4 ± 0.8	2.5 ± 2.8	4.5 ± 2.7	4.3 ± 0.6	4 ± 1.2	3.6 ± 1.5	4.7 ± 2.5	4.2 ± 0.4	3.2 ± 0.7	2.9 ± 0.5	2.7 ± 0.7	3.4 ± 0.3
Isobutiric acid	9.6 ± 0.8	6.1 ± 2	6.2 ± 1.8	9.6 ± 0.4	7.7 ± 1.2	9.5 ± 0.6	8.2 ± 1.2	10.5 ± 0.8	6.9 ± 1.6	7.4 ± 1.5	7.6 ± 0.6	7.9 ± 0.8
Butiric acid	0.2 ± 0.1	0.3 ± 0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0	0.5 ± 0	0.7 ± 0	0.6 ± 0	0.2 ± 0	0.4 ± 0	0.5 ± 0.1	0.5 ± 0
3-methylbutanoic acid	1.3 ± 0	1.1 ± 0.2	1.4 ± 0.1	1.2 ± 0.1	1.1 ± 0	1 ± 0.1	1 ± 0.1	1.1 ± 0	1 ± 0.1	1 ± 0	1 ± 0.1	1 ± 0
Pentanoic acid	0.4 ± 0	0.3 ± 0.1	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0.1	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.2 ± 0
Hexanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Octanoic acid	0.6 ± 0	0.5 ± 0	0.7 ± 0.1	0.7 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.5 ± 0	0.5 ± 0
Decanoic acid	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.2 ± 0	n.d.	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0
Dodecanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SCFA	17 ± 1.1	10.2 ± 5	12.8 ± 3.8	15.8 ± 0.9	13.6 ± 2	14.8 ± 1.7	14.8 ± 3.1	16.6 ± 1.2	11.5 ± 2.4	11.9 ± 1.9	11.9 ± 1.1	13 ± 1.2
MCFA	0.8 ± 0	0.6 ± 0	1 ± 0.1	0.9 ± 0	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0	0.7 ± 0	0.5 ± 0	0.7 ± 0	0.7 ± 0.1	0.7 ± 0
FA	17.7 ± 1.1	10.8 ± 5	13.8 ± 3.7	16.7 ± 0.9	14 ± 2	15.3 ± 1.7	15.3 ± 3.1	17.3 ± 1.2	12.1 ± 2.4	12.5 ± 1.9	12.6 ± 1	13.6 ± 1.2
Ethyl hexanoate	1.6 ± 0.4	3.1 ± 0.7	2.9 ± 0.2	1.3 ± 0	3.8 ± 0.5	2.8 ± 0.3	0.6 ± 0.3	0.5 ± 0.2	1 ± 0	2.6 ± 0.4	1.1 ± 0.1	1.1 ± 0.2
Ethyl octanoate	1.1 ± 0	1 ± 0.2	1.2 ± 0.1	0.8 ± 0	1.2 ± 0.1	1 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	1 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
Ethyl butanoate	3.1 ± 0.2	0.1 ± 0.1	0.2 ± 0	0.1 ± 0.1	0.1 ± 0	0.2 ± 0.1	5.8 ± 0.3	0.1 ± 0	4.3 ± 0.2	2.2 ± 0.4	0.1 ± 0	3 ± 0.2
Ethyl lactate	n.d.	96.4 ± 10	97.1 ± 3.2	25.7 ± 5.7	7.4 ± 0.1	96.1 ± 5.8	95.7 ± 7.2	93.7 ± 4.5	8.6 ± 0.3	91.3 ± 2.9	108.1 ± 4.1	75 ± 3.3
Ethyl decanoate	n.d.	0.4 ± 0	n.d.	n.d.	0.6 ± 0.2	0.6 ± 0	0.9 ± 0.4	1 ± 0.1	n.d.	n.d.	n.d.	n.d.
Diethyl succinate	n.d.	0.2 ± 0	0.2 ± 0	0.2 ± 0.1	0.1 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0	n.d.	0.3 ± 0.1	0.2 ± 0	0.2 ± 0
Ethyl dodecanoate	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
Ethyl esters of FA	6.1 ± 0.4	4.9 ± 0.8	4.7 ± 0.1	2.6 ± 0.1	6 ± 0.7	4.8 ± 0.4	8.5 ± 0.2	2.8 ± 0.4	6.6 ± 0.2	6 ± 0.3	2.2 ± 0.1	5.2 ± 0.4
2-methyl-propanol	60.5 ± 3	56.2 ± 7.9	50.2 ± 11.6	57.3 ± 5.1	62.1 ± 4.8	66.6 ± 6	57 ± 3.4	65.2 ± 1.4	64.8 ± 7.2	55.2 ± 2.1	55.5 ± 8.5	59.4 ± 3
1-butanol	1 ± 0	1.4 ± 0.1	0.7 ± 0.1	1.1 ± 0	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.3	1.5 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
2- methyl-1-butanol	108.5 ± 2.1	102.2 ± 7.6	122.2 ± 5.1	104.2 ± 4.5	109 ± 1.3	96.4 ± 6.2	103 ± 9.1	107.3 ± 1.2	101.9 ± 2.7	104.9 ± 7.9	100.6 ± 6.9	102.4 ± 2.6

Phenylmethanol	3 ± 0.3	2.9 ± 0.3	3.1 ± 0.5	3 ± 0.3	4.5 ± 0.1	4.6 ± 0.2	4.3 ± 0.1	4.9 ± 0.2	3.9 ± 0.7	3.9 ± 0.9	3.8 ± 0.6	3.9 ± 0.2
2-phenylethanol	52.3 ± 1.6	67.4 ± 5	62.7 ± 1.6	54.6 ± 2.4	70.5 ± 3.5	69.5 ± 2.7	66.5 ± 3.3	72.7 ± 2.2	60.9 ± 2.2	68.5 ± 4.1	64.3 ± 1.2	62.9 ± 2
1-propanol	n.d.	15.8 ± 3.8	13.3 ± 5.2	15.4 ± 1.4	n.d.	18.9 ± 9.8	2.3 ± 0.7	24 ± 1.9	n.d.	1.2 ± 0.2	16.5 ± 1.9	0.3 ± 0.1
1-pentanol	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0	0.1 ± 0	0.1 ± 0.1	n.d.	0.1 ± 0	0.1 ± 0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0	0.1 ± 0
1-hexanol	2.3 ± 0.1	0.8 ± 0.1	2.5 ± 0.2	2.3 ± 0.1	1.7 ± 0	1.6 ± 0	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.6 ± 0.2	1.8 ± 0	1.8 ± 0.1
cis-3-hexen-1-ol	0.2 ± 0	0.2 ± 0.1	0.2 ± 0	0.2 ± 0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0	0.3 ± 0.1	0.3 ± 0	0.2 ± 0	0.2 ± 0
Fusel alcohols	228 ± 5.9	247.1 ± 16.8	254.9 ± 18.9	238.1 ± 12.4	249.8 ± 7.6	259.5 ± 18.6	236.9 ± 13.6	277.6 ± 4.8	235.3 ± 12.6	236.9 ± 12.8	244.2 ± 16.3	232.2 ± 7.7
2-phenylethanol acetate	5 ± 0.1	3.1 ± 0.6	3.6 ± 0.5	3.2 ± 0.1	4.7 ± 0.2	3.9 ± 0.2	3.3 ± 0	3.3 ± 0.1	4.5 ± 0.3	3.6 ± 0.5	3.3 ± 0.3	2.9 ± 0.1
Isobutil acetate	0.2 ± 0	0.2 ± 0	0.3 ± 0.1	0.1 ± 0	0.3 ± 0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0
Isoamil acetate	0.3 ± 0	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0	0.3 ± 0	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0	0.4 ± 0.2	0.4 ± 0.1
Fusel alcohols acetates	5.5 ± 0.1	3.6 ± 0.7	4.3 ± 0.6	3.7 ± 0.2	5.3 ± 0.1	4.3 ± 0.3	3.9 ± 0.2	3.8 ± 0.2	5.1 ± 0.1	4.1 ± 0.5	3.8 ± 0.5	3.5 ± 0.1
Isopropanol	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	0.6 ± 0	0.4 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
2-butanol	2.5 ± 0.1	5.5 ± 0.7	3.1 ± 0.4	2.5 ± 0.3	6.5 ± 0.2	5.7 ± 0.7	5.3 ± 0.1	6 ± 0.2	4.6 ± 0.1	4.3 ± 0.2	4.5 ± 0.8	4.8 ± 0.3
1-octanol	1 ± 0.2	1.4 ± 0.1	1.3 ± 0.2	1.4 ± 0.2	n.d.	0.8 ± 0.1	1 ± 0.2	1.2 ± 0.1	0.7 ± 0.1	1 ± 0.1	0.9 ± 0	1.1 ± 0.1

CHAPTER II

Malolactic fermentation and population modulation of a *Oenococcus oeni* defined community by *Torulaspora delbrueckii*

Aitor Balmaseda[a], Nicolas Rozès[b], Albert Bordons[a],

Cristina Reguant[a,*]

* Corresponding author: cristina.reguant@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

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Abstract

Background and Aims: The use of *T. delbrueckii* as starter of the alcoholic fermentation (AF) is increasing due to its chemical modulation in wine. Previous works with this yeast in natural must showed a different *Oenococcus oeni* population by the end of malolactic fermentation (MLF). In this study we aimed to evaluate this aspect in a defined *O. oeni* strain consortium in sterile grape must during winemaking.

Methods and Results: Before performing an alcoholic fermentation with *S. cerevisiae* or both *T. delbrueckii*/ *S. cerevisiae*, the must was inoculated with a defined population of *O. oeni* strains. The use of *T. delbrueckii* determined the bacteria population at the end of MLF. Also, the inoculation of a selected strain after AF produced wines with different organoleptic characteristics to those fermented with the initial bacteria community.

Conclusions: The use of different yeast inoculation strategies modulates the *O. oeni* population and this impacts in the chemical composition of wines. Moreover, the inoculation of a little *O. oeni* population in must leads to a process similar to a spontaneous MLF.

Significance of the Study: *T. delbrueckii* can be used as a tool to modulate the *O. oeni* population and enhance aromas related with MLF.

Keywords

Oenococcus oeni, non-*Saccharomyces*, population dynamics, malolactic fermentation, volatile compounds

Introduction

Oenococcus oeni is the main species of lactic acid bacteria (LAB) that carries out the malolactic fermentation (MLF) in alcoholic fermented beverages as wine and cider (Lonvaud-Funel, 1999; Wibowo et al., 1985). MLF consists in the decarboxylation of L-malic acid into L-lactic acid, which is related with a pH increase, microbial stability improvement and production of aroma compounds (Lonvaud-Funel, 1999). This metabolism is key for LAB survival under the stressful conditions found in wine as low pH, high ethanol concentration and low nutrient availably (Bech-Terkilsen et al., 2020).

These LAB that participate in the MLF come from the grapes, must and also can be part of the resident microbiota of the cellar (Franquès et al., 2017; González-Arenzana et al., 2012a; Portillo et al., 2016). As grapes are transformed in must and then, into wine; the LAB population becomes more restricted, and the main significant species is *O. oeni*. Besides, the dominance of this bacterial species often is not achieved with just a single of few strains; it can be formed by several dominant strains, which can be successively modified (Reguant et al., 2005b).

Under this oenological context, the population of *O. oeni* will be greatly affected by the grape variety (Portillo et al., 2016) and berries health status (Lleixà et al., 2018), the vinification place (González-Arenzana et al., 2013) and, of course by the fermenting yeasts that undergo the alcoholic fermentation (AF). In this final point the selected yeasts strains inoculated in must to undergo the AF have a considerable impact (Alexandre et al., 2004; Balmaseda et al., 2018). Traditionally *S. cerevisiae* has been used as starter culture in winemaking (Fleet, 2008). Nevertheless, current research in non-*Saccharomyces* yeasts, related with the first fermentative stages, proposes the use of these species to modulate the chemical and organoleptic characteristics of wines (Padilla et al., 2016b). Besides, these non-*Saccharomyces* yeasts can have an impact upon the *O. oeni* community developed in those wines due to that chemical modulation (Balmaseda et al., 2018).

T. delbrueckii is a non-*Saccharomyces* which has been proposed as a microbial tool to improve wine characteristics (Benito, 2018a). It is of special interest in the red winemaking due to an enhancement of the color parameters and volatile compounds

(Belda et al., 2017b; Escribano-Viana et al., 2019). Moreover, it reduces the concentration of various compounds related with inhibitory effect upon *O. oeni* as ethanol, SO_2, succinic acid, etc. and promotes some stimulatory changes, such as the increase in mannoproteins concentration and in pH (Belda et al., 2016; Benito, 2018a; Ferrando et al., 2020; Martín-García et al., 2020). Additionally, recent studies reported differences in the *O. oeni* strain imposition at the end of MLF associated to the use of *T. delbrueckii* when compared to wines only fermented with *S. cerevisiae* (**Chapter I: 1, 2**). This fact reinforces the important concept of yeast- *O. oeni* strain compatibility for a successful MLF performance. Also, the use of *T. delbrueckii* enabled the spontaneous MLF in high polyphenolic red wines, not possible in *S. cerevisiae* wine (**Chapter I: 2**).

In this present work we aimed to evaluate the influence of using *T. delbrueckii* in the evolution of a defined community of *O. oeni* strains. For this purpose, we inoculated a sterile must with a selection of *O. oeni* strains and underwent the AF with *T. delbrueckii* in sequential inoculation with *S. cerevisiae*. The impact of the inoculation strategy in the *O. oeni* community was evaluated by the end of MLF and some relevant oenological parameters throughout the fermentative process were studied.

Materials and methods

Microorganisms

The yeast strains used were *T. delbrueckii* Biodiva (Lallemand Inc., Montréal, Canada) (TdB), *T. delbrueckii* Viniferm NS-TD (Agrovin S.A., Spain) (TdV) and *S. cerevisiae* Lalvin-QA23 (Lallemand Inc.) (Sc). For the defined *O. oeni* community, referred from this point as consortium, four strains isolated from wines fermented with *T. delbrueckii* in previous works were used together with the commercial strain Viniflora-CH11 (Chr. Hansen S.L.). These four strains were isolated from vintage 2018 – M25, MCS5 – (**Chapter I: 1**) and vintage 2019 – AiB9, AiB14 – (**Chapter I: 2**). Besides, *O. oeni* CH11 showed a better MLF performance in *T. delbrueckii* fermented wines in those previous works. As starter culture of MLF after AF, *O. oeni* PSU-1

(ATCC BAA-331) (PSU-1), was selected. The experimental design of the inoculation strategies is represented in Figure 16.

Yeasts were maintained on YPD plates (2% glucose, 2% bacto-peptone, 1% yeast extract, 2% agar, w/v, Panreac Química SLU, Castellar del Vallès, Spain) and bacteria on MRSmf plates (Martín-García et al., 2020), and all of them were stored at 4 °C.

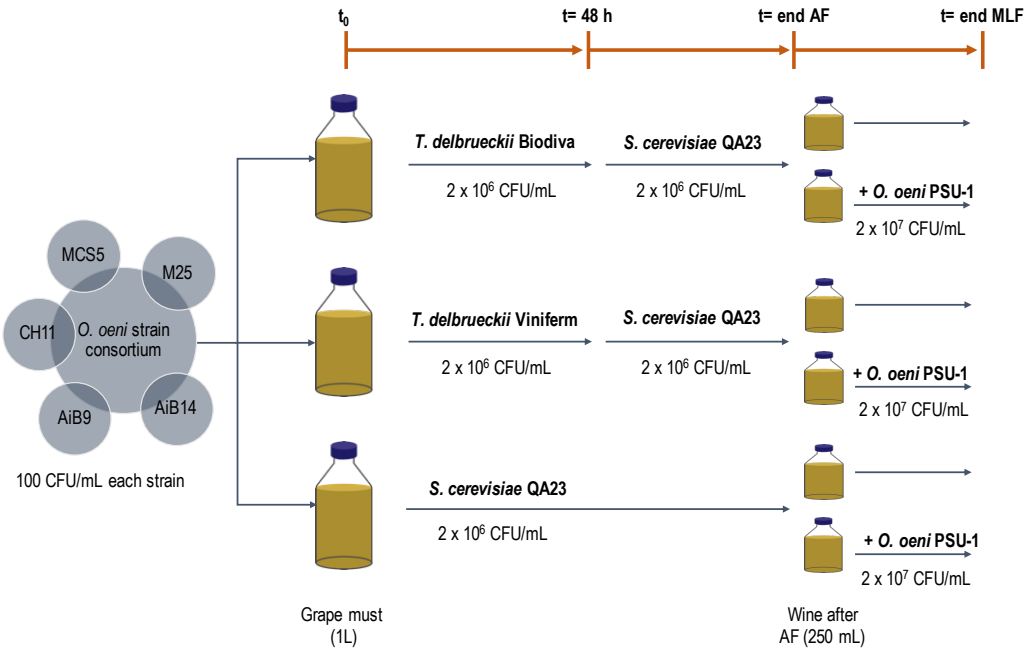

Figure 16. Schematic representation of the inoculation strategies used in this work.

Experimental fermentations

Fermentations were performed in natural concentrated must (Mostos S.A., Tomelloso, Spain) diluted with sterile MiliQ water until a density of 1075 ± 1 g/L (Martín-García et al., 2020), which corresponded to an initial concentration of citric acid and L-malic acid of 0.32 g/L and 2 g/L respectively. Must was supplemented with 0.4 g/L of Nutrient Vit NatureTM (Lallemand Inc., Montreal, Canada) and pH was adjusted to 3.6. Then, must was sterilized using 0.1% (v/v) of dimethyl dicarbonate (ChemCruz®, USA) and stored overnight at 4 ºC.

Fermentations were carried out in 1 L flasks containing 1 L of must statically at 20 ºC in triplicate. First, grape must was inoculated with the *O. oeni* consortium. Each strain was grown separately to a population of around 10^9 CFU/mL in MRSmf liquid medium. Then, equal populations of each strain were added to sterile saline solution 0.9 % (w/v). The resulting mixture was serially diluted in saline solution and added to

each must replicate for a theoretical total population of 500 CFU/mL, corresponding to around 100 CFU/mL for each strain. The intention was to emulate the indigenous LAB population levels found in must, which may oscillate between 10^2 and 10^4 CFU/mL (Lonvaud-Funel, 1999).

Yeasts were inoculated for a population of 2 x 10^6 cell/mL to undergo the AF. In the case of sequential inoculation with *T. delbrueckii*, after 48 h of initial inoculation, *S. cerevisiae* QA23 was inoculated for the same population. The decrease in density and yeast population were determined at least every 48h. YPD agar medium was used to count the total viable yeasts and lysine agar medium (Oxoid LTD., Basingstoke, UK) for the enumeration of non-*Saccharomyces*, after incubation at 28 ºC for 48 h. AF was considered to have finished when the sugar concentration was below 2 g/L.

At this point, 250 mL of each wine was transferred into two sterile 250 mL flasks. One of the flasks of each wine was inoculated with *O. oeni* PSU-1 for a population of 2 x 10^7 CFU/mL in order to evaluate the impact of the use of a MLF starter on the evolution of the *O. oeni* consortium. The other flask of each wine was not modified Then, the two flasks were incubated in the same conditions as AFs. These MLFs were also carried out in triplicate. Samples were taken every 24 h to monitor the consumption of L-malic acid and the evolution of the bacterial population in the wines inoculated with PSU-1 and more spaced in the others, which only contained the bacterial consortium. Samples were plated on MRSmf and incubated at 27 ºC in a 10 % CO_2 atmosphere for 7–15 days. MLF was considered to have finished when the L-malic acid was below 0.1 g/L.

O. oeni typification

10 isolates at the end of the inoculated wines and 20 from the non-inoculated wines were randomly selected from MRSmf plates for typing. **Chapter I: 1** was followed for DNA extraction and typing procedure based on VNTR method described by Claisse & Lonvaud-Funel (2014). Samples were analysed using capillary electrophoresis by Eurofins Genomics Europe (Edersberg, Germany).

Wine characterization

Sugar content at final stages of AF and L-malic acid during MLF were determined using the multianalyser Miura One (TDI SL, Gavà, Spain). On completion of AF and MLF, pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain).

Mannoprotein content of WLM before and after MLF was quantified using D-mannose and D-glucose assay kit K-MANGL (Megazyme, Wicklow, Ireland). Mannose corresponding to mannoproteins in wine were extracted as described in **Chapter III: 1**.

Succinic acid after AF was determined using the Succinic Acid Assay Kit K-SUCC (Megazyme, Wicklow, Ireland). Glucose, glycerol, acetic acid, citric acid, lactic acid and ethanol of wines after AF and MLF were determined by high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) as described in Zhu, Navarro, Mas, Torija, & Beltran (2020).

Wine volatile compound analysis

Wine samples (10 mL) were taken after AF and MLF. The volatile compounds were liquid/liquid extracted with 0.4 mL dichloromethane and 2.5 g $(NH_4)_2SO_4$ adding 40 µL of a solution of 4-methyl-2-pentanol (0.8 g/L) and heptanoic acid (0.7 g/L) as internal standards. Samples were analysed as described in **Chapter I: 1**.

Statistical analysis

All the statistical analyses of the results were performed using the statistics software XLSTAT version 2020.2.3.65345 (Addinsoft, Paris, France). To test for differences among samples, a one-way ANOVA was performed using Tukey's post hoc HSD test at a *p*-value of 0.05.

Results and discussion

Fermentations and microbial growth parameters

The duration of AF was dependent of the inoculation strategy used (Figure 17). As usual, the sequential inoculation with non-*Saccharomyces* increased the time of the AF

as it has been reported in synthetic or natural media (**Chapter I: 1**; Martín-García et al., 2020). Sequential inoculation with *T. delbrueckii* increased the AF duration around a 50% regarding to *S. cerevisiae* control fermentation (Figure 17, Table 11). During the fermentative process, the two *T. delbrueckii* strains remained viable until the middle fermentation stage. Afterwards, their viability was lost in Lys plates (< 10^4 CFU/mL). After AF, as yeast lees were maintained in wine, they continued to be viable during MLF. Indeed, the viable yeast population remained around 2-4 x 10^5 CFU/mL in all wines, including those not inoculated with *O. oeni* PSU-1 (data not shown).

The *O. oeni* consortium evolved differently depending on the yeast inoculation strategy used for AF (data not shown). From the calculated 500 CFU/mL inoculated, after an 1h of incubation the detected population in all wines was of around 300 CFU/mL. After 2 days of *S. cerevisiae* inoculation in Sc wines, the bacterial viable population was undetectable in MRSmf plating. Contrary, after 2 days of *T. delbrueckii* fermentation in TdB and TdV wines, the population of *O. oeni* consortium was maintained around 300 CFU/mL. Nevertheless, at the fermenting day 4 – two days after *S. cerevisiae* inoculation – the viable population was undetectable in these wines. This clearly shows a positive interaction between *T. delbrueckii* and *O. oeni* that allowed the maintenance of the bacterial consortium population during the two first days. Probably, the high fermentative capacity of *S. cerevisiae* resulted in the viability loss of the consortium when it was inoculated. This can be observed in the density decrease (Figure 17). *T. delbrueckii* underwent a less active fermentative process than *S. cerevisiae*, which can be related to more gradual changes that would allow *O. oeni* to keep viable, although this did not support bacterial growth.

No many differences were observed in the global fermentative process due to the use of *T. delbrueckii* in the MLFs inoculated with *O. oeni* PSU-1 (Table 11). As result of an extended duration in *T. delbrueckii* AF and similar MLF duration, the total fermentative process was longer in *T. delbrueckii* wines. Among these fermentations, the one of TdV wine resulted in the longest fermentation with the least consumption rate and the lowest maximum biomass (Table 11). No significant differences were observed in Sc and TdB wines.

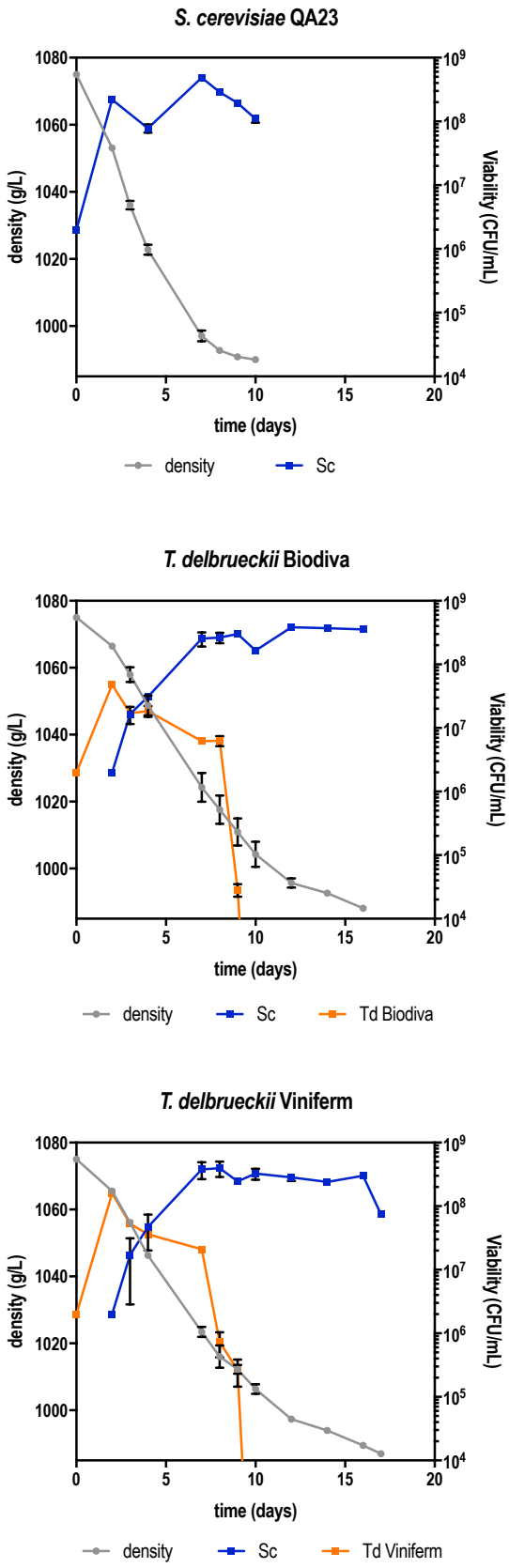

Figure 17. Evolution of alcoholic fermentations by monitoring density decrease and yeast cell viability. Sc, Td Biodiva and Td Viniferm correspond to *S. cerevisiae*, *T. delbrueckii* Biodiva and *T. delbrueckii* Viniferm. (-●-) density, (-■-) *S. cerevisiae*, (-■-) *T. delbrueckii*.

Table 11. Fermentative and growth parameters of alcoholic and malolactic fermentations. Values shown are the mean of triplicates ± SD. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva and *S. cerevisiae*), TdV (*T. delbrueckii* Viniferm and *S. cerevisiae*) fermented wines.

		AF duration (days)	MLF lag phase (days)	MLF (days)	Total duration (days)	Consumption rate+ (L-malic acid g/L·day)	Growth phase* (days)	Maximum biomass (CFU/mL)	Growth rate (day^{-1})
PSU-1	Sc	10 ± 0	n.d.^{a}	4 ± 0^{a}	14 ± 0^{a}	0.7 ± 0.15^{b}	n.d.^{a}	6.3 x 10^{7} ± 6.7 x 10^{6} ^{a}	
	TdB	16 ± 0	n.d.^{a}	4 ± 0^{a}	20 ± 0^{a}	0.54 ± 0.04^{b}	n.d.^{a}	9.6 x 10^{7} ± 9 x 10^{6} ^{ab}	
	TdV	17 ± 0	n.d.^{a}	5 ± 0^{a}	22 ± 0^{a}	0.25 ± 0.01^{a}	n.d.^{a}	3.7 x 10^{7} ± 4.8 x 10^{6} ^{a}	
Consortium	Sc	10 ± 0	66 ± 3^{b}	11 ± 1^{c}	87 ± 4^{b}	0.19 ± 0.01^{a}	20 ± 1^{c}	7.6 x 10^{7} ± 3.3 x 10^{7} ^{a}	0.46 ± 0.01^{a}
	TdB	16 ± 0	70.5 ± 1^{b}	8.5 ± 1^{b}	95 ± 2^{b}	0.12 ± 0.01^{a}	14 ± 1^{b}	5 x 10^{8} ± 4 x 10^{8} ^{b}	0.58 ± 0.01^{b}
	TdV	17 ± 0	87 ± 31^{b}	7.5 ± 1^{b}	111.5 ± 32^{b}	0.24 ± 0.07^{a}	19 ± 4^{c}	2.7 x 10^{8} ± 2.7 x 10^{8} ^{ab}	0.47 ± 0.03^{a}

+ Calculation based on consumption rate of L-malic acid considering the period of exponential decrease of these values. * Growth phase refers to the time needed to reach sufficient *O. oeni* population to start consuming L-malic acid since viable cells were detected. $^{a–c}$Indicate significant differences at $p \leq 0.05$ according to a Tukey post-hoc comparison test. n.d.: not detected.

The MLFs undergone by the consortium, without *O. oeni* inoculation after AF, had large lag phases (Table 11). Usually, spontaneous MLF need long time to reach enough population, around 10^{5} CFU/mL, to start L-malic consumption (Reguant et al., 2005b). Interestingly, there was no statistical differences in the observed lag phases due to the use of different yeasts (Table 11). Nevertheless, the high heterogeneity of the results achieved in TdV wine, masked that one of the replicates of this wine had a lag phase of 65 days, similar to the other average lag phases in Sc and TdB wines. The impact of the use of *T. delbrueckii* was noticeable in MLF duration, considered as the time employed in L-malic acid consumption, disregarding the initial lag phase (Table 11). In this sense, the use of both *T. delbrueckii* strains reduced the time of MLF fermentation. This is in accordance with the previously reported effect of this yeast on MLF when *O. oeni* is inoculated or a reduction of the time of spontaneous fermentation is observed (**Chapter I: 2**).

O. oeni strain population at the end of MLF

To understand the impact of *T. delbrueckii* in *O. oeni* strain population diversity we defined the consortium with five strains: four autochthonous *O. oeni* strains isolated from *T. delbrueckii* fermented wines and one commercial strain CH11 with enhanced

MLF performance in *T. delbrueckii* wines (**Chapter I: 1, 2**). Moreover, we inoculated PSU-1 after AF to have a control condition in which *O. oeni* is inoculated in wine after AF.

The dynamics of *O. oeni* strains population differed in the wines as consequence of the yeast inoculation strategy used (Figure 18). Two tendencies were observed in bacterial population at the end of MLF. One clustered the population of Sc and TdB wines. The other corresponded to TdV wines.

In Sc and TdB wines, the imposition of PSU-1 was total, no other strain coming from the initial consortium was detected (Figure 18). In previous studies we have observed the capacity of imposition of PSU-1 when used as MLF starter culture in the cellar (**Chapter I: 1, 2**). In those wines without PSU-1 inoculation, the dominant strain from the consortium at the end of MLF was the commercial CH11, which corresponded up to 80% in TdB wine and 90% in Sc wine in average (Figure 18). The other only strain detected in these wines was AiB9. The presence of this particular strain was significantly greater in TdB wine compared to Sc wine, suggesting that *T. delbrueckii* can favour *O. oeni* diversity (**Chapter I: 1**).

In TdV wine it was observed a different pattern (Figure 18). First, in the wine inoculated with PSU-1, the strain MSC5 was detected. Contrary to the other wines, in TdV wine the dominance of PSU-1 was not complete and approximately a 15% in average of the detected population was MCS5. The wine not inoculated with PSU-1 presented a different population at the end of MLF than in Sc and TdB wines. Surprisingly, the presence of CH11 was poor by representing just the 20% in average. In this wine, the dominant strain corresponded to M25.

Altogether, although both *T. delbrueckii* strains showed a similar persistence during AF in sequential inoculation with *S. cerevisiae* QA23, the use of Biodiva strain did not significantly modify the *O. oeni* population behaviour compared to the control with only *S. cerevisiae* QA23, whereas Viniferm strain completely changed it. When we relate these data with MLF performance (Table 11), not many correlations can be pointed. Nevertheless, it is interesting to comment that the growth rate associated with TdB wine was significantly higher than the one of Sc wine, being composed by the same *O. oeni* population. Also, the duration of MLF, only considering the exponential

consumption of L-malic acid, increased when the percentage of imposition of CH11 decreased. Some authors have reported different L-malic consumption rates by different *O. oeni* strains, which can be related (Nehme et al., 2010). Still, MLF is a complex microbial process where the dominant strains may change along time, so other strains could have participated in the fermentation apart from those detected at the end of MLF.

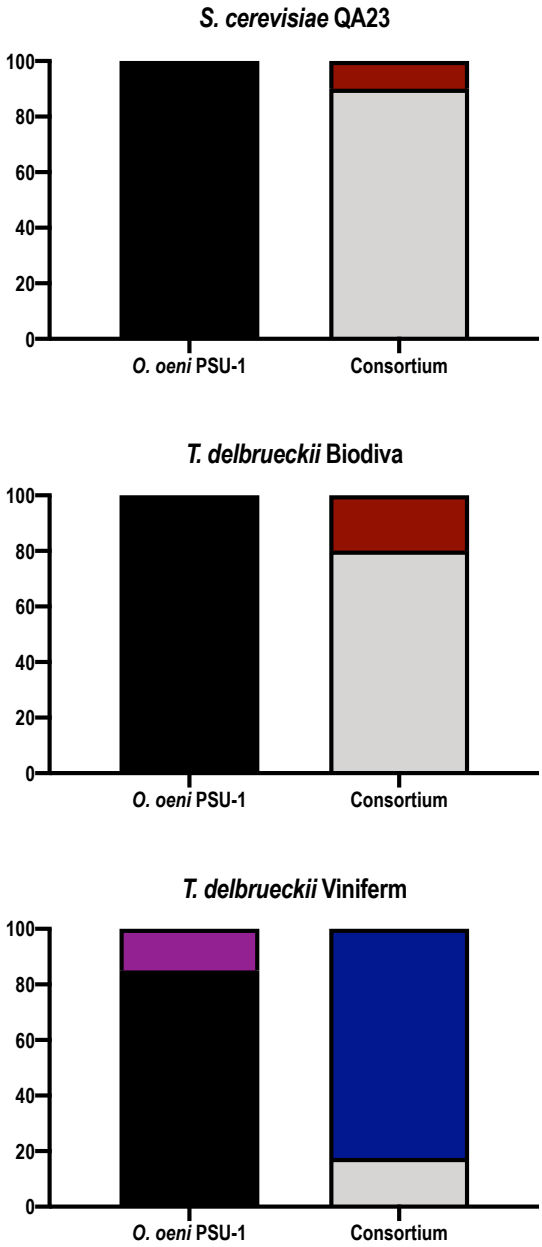

Figure 18. Percentage of imposition of the different VNTR profiles of *O. oeni* after malolactic fermentation in wines inoculated after AF (PSU-1) or fermented with the initial bacterial population (consortium). PSU-1 refers to *O. oeni* PSU-1, which was inoculated after AF. CH11, AiB9, MCS5 and M25 correspond to the *O. oeni* strains inoculated in the initial must. (■) PSU-1, (■) CH11, (■) AiB9, (■) MCS5, (■) M25.

In spontaneous MLF or wines inoculated after AF we usually observe higher *O. oeni* strain diversity at the end of MLF. This particular study was performed using a synthetic community with 5 different genotypes which resulted in 1-2 strains by the end of MLF. We point that if the initial community was larger, more strains would have been detected as it occurs in spontaneous fermentations.

Wine chemical composition

The chemical composition of the obtained wines was dependent on the inoculation strategy used (Table 12). Ethanol concentration had a similar concentration of 10.5 % (vol/vol) in all wines, after AF and MLF.

The substrate of the MLF, L-malic acid, was significantly reduced (around 0.2-0.3 g/L in average) when the two *T. delbrueckii* strains were inoculated (Table 12). In *T. delbrueckii* wines after the inoculated MLF, higher concentration of lactic acid was observed (Table 12) regarding *S. cerevisiae* control wine. This could be related with the traces of glucose and fructose detected in *T. delbrueckii* wines after AF (data not shown). Besides consuming L-malic acid, *O. oeni* can metabolize sugar traces found in wine increasing D-lactic acid (Lonvaud-Funel, 1999). Consequently, we can observe an increase in these wines due to the contribution of D-lactic acid isomer to the total lactic acid concentration.

Citric acid concentration was similar in wines after AF (Table 12). Non-*Saccharomyces* modulation of this particular acid is very heterogeneous since some species increase it (Ferrando et al., 2020) and others produce similar quantities as *S. cerevisiae* (Belda et al., 2017b; Martín-García et al., 2020). Under oenological conditions *O. oeni* metabolize this acid as energy source and its concentration is reduced by the end of MLF (Davis et al., 1986). It is interesting to point that this consumption was observed in the inoculated wines after AF and not in the case of the MLF carried out by the consortium (Table 12). The consumption of citric acid by *O. oeni* is reported in literature as strain specific and generally used when L-malic acid is depleted (Bartowsky and Henschke, 2004). In this sense, it seems that under the studied conditions, the *O. oeni* strains from the consortium exhibited a low

metabolization of citric acid (Table 12), even if the fermentation duration was much longer (Table 11).

Acetic acid is a volatile compound which can be produced by yeasts during AF and during MLF as result of citric acid or other sugar consumption (Davis et al., 1986). Similar values were observed after AF and a little increase after MLF mainly in Sc wine with *O. oeni* PSU-1 (Table 12). Acetic acid is one possible compound produced as consequence of citric acid consumption (Bartowsky and Henschke, 2004). Thus, those fermentations that exhibited higher consumption of citric acid, as observed when inoculating *O. oeni* PSU-1, could contribute to increase acetic acid concentration. Moreover, the little increase after MLF in the consortium wines, after a long period of incubation, could be related with those remaining metabolically active yeast lees.

Succinic acid, which is a competitive inhibitor of L-malic acid for the active site of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982) can be regarded as a potential inhibitor of MLF in wine. Some previous studies have related the use of *T. delbrueckii* with a decrease in succinic acid concentration (**Chapter I: 1**; Martín-García et al., 2020). In concordance with those studies, in this experiment we observed a significant decrease in the sequential inoculations with *T. delbrueckii* (Table 12). Nevertheless, the detected concentrations are still quite low to directly impact in MLF performance.

Glycerol was increased by the use of *T. delbrueckii* in wines after AF (Table 12). Indeed, this yeast is usually related with higher production of glycerol than *S. cerevisiae* under oenological conditions (Belda et al., 2016; González-Royo et al., 2015). Also, a little significant increase in this compound was observed after MLF in Sc and TdB wines. To best of our knowledge there is no published study that states an increase in glycerol after MLF. Nevertheless, the higher concentration of this compound should respond to the release of it from yeast lees since no direct relation with *O. oeni* is known. Thus, we can suggest with these results that fermenting over yeast lees can contribute to increase glycerol concentration in some cases.

Table 12. Oenological parameters and some volatile compound families of wines after alcoholic and malolactic fermentations. Values shown are the means of triplicates ± SD. Sc, TdB and TdV correspond to *S. cerevisiae*, *T. delbrueckii* Biodiva- *S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* fermented wines, respectively.

	L-malic acid (g/L)	Lactic acid (g/L)	Citric acid (g/L)	Acetic acid (g/L)	Succinic acid (mg/L)	Ethanol (% vol/vol)	Glycerol (g/L)	pH	Σ SCFA (mg/L)	Σ MCFA (mg/L)	Σ Fusel alcohol (mg/L)	Σ Fusel alcohol acetates (mg/L)
Sc												
AF	1.51 ± 0.1^{c}	n.d.^{a}	0.68 ± 0.03^{d}	0.30 ± 0.04^{ab}	375 ± 4^{c}	10.5 ± 0.2^{a}	4.18 ± 0.67^{a}	3.42 ± 0.03^{a}	2.72 ± 0.32^{ab}	24.57 ± 0.94^{e}	94.43± 0.9^{a}	4.19 ± 0.45^{ab}
PSU-1	n.d.^{a}	1.45 ± 0.01^{b}	0.19 ± 0.02^{a}	0.48 ± 0.07^{d}		10.7 ± 0.1^{a}	6.03 ± 0.07^{bcd}	3.65 ± 0.01^{bcd}	4.01 ± 0.08^{bc}	22.66 ± 0.46^{e}	94.35 ± 2^{a}	3.73 ± 0.3^{a}
Consortium	n.d.^{a}	1.40 ± 0.01^{b}	0.54 ± 0.1^{c}	0.31 ± 0.01^{abc}		10.4 ± 0.1^{a}	5.83 ± 0.06^{bc}	3.59 ± 0.03^{b}	5.5 ± 1.51^{c}	23.97 ± 0.12^{e}	97.03 ± 6.3^{a}	4.44 ± 1^{ab}
TdB												
AF	1.35 ± 0.01^{b}	n.d.^{a}	0.76 ± 0.0^{d}	0.24 ± 0.04^{a}	197 ± 8^{b}	10.2 ± 0.2^{a}	5.26 ± 0.04^{b}	3.57 ± 0.0^{b}	1.06 ± 0.34^{a}	6.92 ± 0.79^{a}	109.57 ± 2.9^{b}	4.25 ± 0.17^{ab}
PSU-1	n.d.^{a}	2.04 ± 0.02^{d}	0.28 ± 0.03^{ab}	0.41 ± 0.01^{cd}		10.5 ± 0.1^{a}	6.15 ± 0.33^{cd}	3.73 ± 0.0^{cd}	4.51 ± 0.34^{bc}	12.8 ± 1.1^{b}	103.09 ± 2.9^{ab}	4.2 ± 0.8^{ab}
Consortium	n.d.^{a}	1.8 ± 0.07^{c}	0.79 ± 0.0^{d}	0.37 ± 0.02^{bcd}		10.2 ± 0.1^{a}	6.02 ± 0.05^{bcd}	3.63 ± 0.09^{bc}	3.82 ± 1.19^{bc}	16.74 ± 0.6^{c}	99. 5 ± 3^{ab}	5.16 ± 1.3^{ab}
TdV												
AF	1.28 ± 0.06^{b}	n.d.^{a}	0.78 ± 0.02^{d}	0.24 ± 0.04^{a}	183 ± 2^{a}	10.5 ± 0.1^{a}	5.97 ± 0.03^{bcd}	3.55 ± 0.0^{b}	3 ± 0.44^{ab}	8.84 ± 0.07^{a}	101.4 ± 4.3^{ab}	5.96 ± 0.7^{bc}
PSU-1	n.d.^{a}	2.04 ± 0.13^{d}	0.32 ± 0.01^{b}	0.39 ± 0.02^{bcd}		10.3 ± 0.3^{a}	6.71 ± 0.18^{d}	3.76 ± 0.01^{d}	3.14 ± 0.16^{ab}	17.45 ± 0.53^{cd}	99.52 ± 3.3^{ab}	7.38 ± 0.52^{c}
Consortium	n.d.^{a}	1.73 ± 0.01^{c}	0.66 ± 0.01^{cd}	0.38 ± 0.02^{bcd}		10.5 ± 0.2^{a}	6.63 ± 0.02^{cd}	3.61 ± 0.08^{bc}	4.66 ± 0.65^{bc}	19.53 ± 1.76^{d}	119.3 ± 4.2^{b}	5.75 ± 0.06^{abc}

$^{a-d}$. Indicate significant differences at $p \leq 0.05$ according to a Tukey post-hoc comparison test.

pH was also dependent on the AF inoculation strategy (Table 12). *T. delbrueckii* wines had slightly significant higher values than the observed in *S. cerevisiae* wine (Martín-García et al., 2020). This is related with better MLF performance since acid pH is one of the most known inhibitor factors for *O. oeni* in wine. Consequence of MLF, pH value increased after MLF. This increase was higher in wines inoculated with PSU-1 than in the consortium fermented wines. This should be related with the yeast lees metabolism that maintained significant viability during all the fermentative process or also to the autolysis process of these lees, which can release intracellular acids to the medium.

Volatile composition of the different wines was quite similar (Table 12). However, some changes were observed. For instance, small chain fatty acids (SCFA) increased after MLF in Sc and TdB wines. Slight differences were noticed at the end of AF in which the wine TdB had the lowest SCFA concentration. In all cases the higher amount of SCFA quantified was consequence of the accumulation of isobutyric acid (Supl. Table 11).

The concentration of medium chain fatty acids (MCFAs) was significantly reduced by the use of *T. delbrueckii* after AF (Table 12). Some authors have already reported a decrease in this family compounds using *T. delbrueckii*. In this study we detected hexanoic (C6), octanoic (C8) and decanoic (C10) acids. C6 was not detected at the end of AF in *T. delbrueckii* wines. This acid was the responsible of the reduction in total MCFA composition (Suppl. Table S5) in TdB and TdV wines. Interestingly, after MLF, the concentration of MCFA was similar in *S. cerevisiae* wines and increased in *T. delbrueckii* wines. Nevertheless, the concentration after MLF in *T. delbrueckii* was never as high as detected in *S. cerevisiae* (Table 12).

Fusel alcohol concentration was significantly higher in TdB wines in regard to *S. cerevisiae* wines (Table 12). Still the increase was not very high to impact in organoleptic profile of wines. Besides, fusel alcohol acetates augmented after MLF (Table 12). The production of fusel alcohol acetates is highly dependent on the enzymatic capacities of the fermenting strains (Ugliano and Moio, 2005) and it is not regulated by the substrate availably (fusel alcohols). After MLF, the increase of this family compounds concentration can depend on the *O. oeni* strain (Ugliano and Moio,

2005). In this sense, as we detected different *O. oeni* strains by the end of MLF (Figure 18) we can relate higher enzymatic capacity with the dominance of M25 strain in this particular medium.

In Figure 19 are represented the changes in a selection of the volatile compounds related with MLF. Ethyl lactate is one of the most abundant volatile compounds produced during MLF. The production of this compound has been related with the inoculated *O. oeni* strain (Malherbe et al., 2012). Our results showed a similar ethyl lactate production in Sc and TdB wines, which had similar *O. oeni* strain composition (Figure 19). Contrary, TdV that was associated with a different fermenting strains at the end of MLF had higher levels in the consortium fermented wines and a little reduction was observed in PSU wine. Thus, we can relate M25 strain with higher production of this compound.

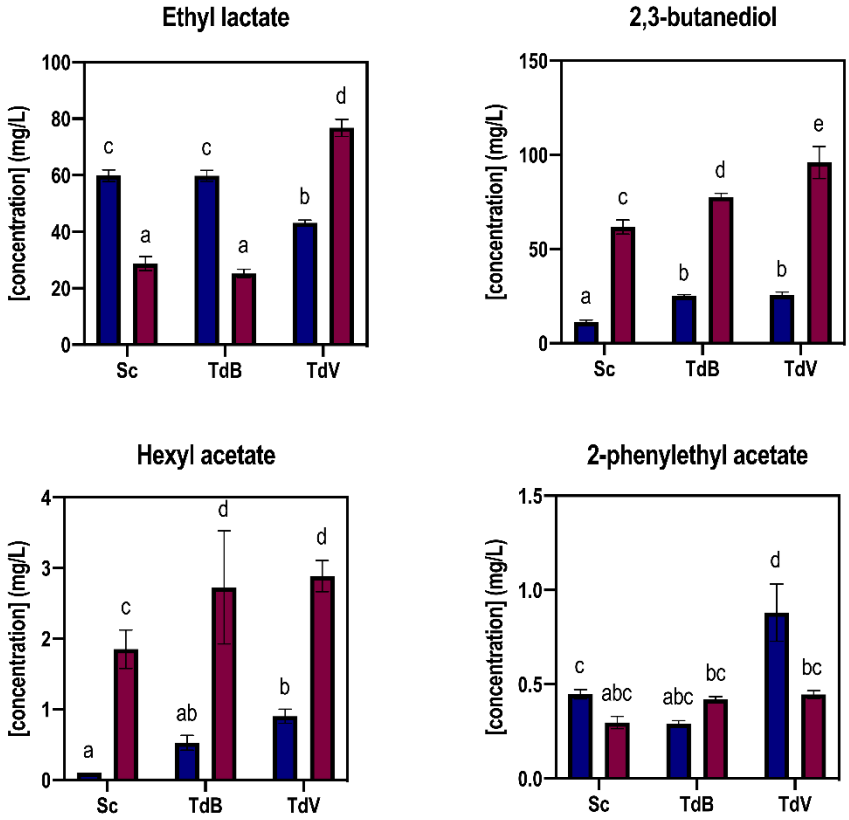

Figure 19. Concentration of some selected aromas related with malolactic fermentation with significant differences. Values shown are the mean of triplicates ± SD. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva and *S. cerevisiae*), TdV (*T. delbrueckii* Viniferm and *S. cerevisiae*) fermented wines. PSU-1 refers to the wines inoculated with that strain after AF. Consortium refers to the wines fermented with the initial bacterial population. (■) PSU-1, (■) consortium.

According to 2,3-butanediol, it was interesting to observe that in the consortium fermented wines, the production of this compound was much higher than in the wines inoculated with PSU-1. This compound can be related with citric acid metabolism by *O. oeni* (Bartowsky and Henschke, 2004). Moreover, wines in contact with yeast lees are related with higher butanediol concentrations (del Fresno et al., 2019). As commented before, in our study yeast viability was maintained during all the fermentative process. As consequence, the metabolically active lees could increase 2,3-butanediol concentration in consortium fermented wines. Besides, significant higher 2,3-butanediol concentration was detected in *T. delbrueckii* wines inoculated after AF, which corresponded to longer yeast lees contact (16 and 17 days) with respect to Sc wine (10 days).

Hexyl acetate concentration was significantly much higher in consortium fermented wines than in wines inoculated with PSU-1 (Figure 19). The production of this compound is usually related to the specific characteristics of the fermenting strain (Malherbe et al., 2012). We observed that the wines inoculated after AF with *O. oeni* PSU-1 had similar and lower values than those fermented with the consortium, which corresponded to another *O. oeni* population. The concentration of 2-phenylethyl acetate usually remains constant after MLF (Malherbe et al., 2012; Pozo-Bayón et al., 2005). Nevertheless, some authors observed an increase after MLF (Ugliano and Moio, 2005). Not many differences were observed in this compound with the exception of one wine. In TdV wine inoculated with PSU-1 the levels of this compound were significantly higher than in the other wines (Figure 19), indeed, doubling the concentration.

Conclusions

In this paper we studied the effect of the inoculation strategy, regarding to *T. delbrueckii* use, during AF in a defined community of *O. oeni* strains. Under these controlled conditions we observed a direct impact in *O. oeni* community regarding to the inoculated yeasts. The dynamics of the *O. oeni* strains population was significantly modified by one of the used *T. delbrueckii* strains, Viniferm. Whereas the other strain, Biodiva, did not modify the *O. oeni* strain evolution when compared to the wines inoculated only with S. *cerevisiae*. Still, even if an effect in population was noticed, not

much impact was observed in the duration of the MLF process. As the community changed, the quantified wine parameters also were modulated. These data were also compared to wines inoculated with a selected *O. oeni* strain at the end of AF. In this sense, the volatile composition of wines fermented with the initial consortium was more complex and aromas related with MLF were enhanced, such as 2,3-butanediol and hexyl acetate. Altogether, these results present new data that highlights the impact of the inoculated yeast in *O. oeni* population, which can be modulated by the use of non-conventional yeasts.

Supplementary Tables

Suppl. Table S5. Composition of volatile compounds (mg/L) in the obtained wines. Values shown are the means of triplicates ± SD. Sc, TdB and TdV correspond to *S. cerevisiae*, *T. delbrueckii* Biodiva- *S. cerevisiae* and *T. delbrueckii* Viniferm-*S. cerevisiae* fermented wines, respectively. P and C correspond to PSU-1 and consortium, respectively. n.d.: not detected

	Sc	TdB	TdV	Sc-P	Sc-C	TdB-P	TdB-C	TdV-P	TdV-C
Propionic acid	0.49 ± 0.01	0.38 ± 0.16	0.65 ± 0.09	0.78 ± 0.02	1.01 ± 0.33	0.64 ± 0.16	0.54 ± 0.15	0.61 ± 0.07	0.77 ± 0.04
Butyric acid	0.12 ± 0.04	0.12 ± 0.05	0.12 ± 0.03	0.09 ± 0.03	0.13 ± 0.06	0.09 ± 0.03	0.13 ± 0.02	0.14 ± 0.04	0.1 ± 0.01
Isobutyric acid	1.39 ± 0.22	n.d.	1.74 ± 0.38	2.5 ± 0.07	3.54 ± 1.03	3.37 ± 0.2	1.81 ± 0.58	2.11 ± 0.12	2.29 ± 0.5
3-methylbutanoic acid	0.21 ± 0.05	0.08 ± 0.02	0.15 ± 0.05	0.19 ± 0.01	0.32 ± 0.13	n.d.	0.89 ± 0.44	n.d.	1.05 ± 0.08
Valeric acid	0.5 ± 0.05	0.48 ± 0.12	0.34 ± 0.06	0.46 ± 0.01	0.5 ± 0.08	0.41 ± 0.08	0.44 ± 0.08	0.29 ± 0.02	0.44 ± 0.05
ΣSCFA	2.72 ± 0.32	1.06 ± 0.34	3 ± 0.44	4.01 ± 0.08	5.5 ± 1.51	4.51 ± 0.34	3.82 ± 1.19	3.14 ± 0.16	4.66 ± 0.65
Hexanoic acid	13.12 ± 1.01	n.d.	n.d.	12.38 ± 0.18	13.37 ± 0.46	7.14 ± 0.17	9.42 ± 0.3	9.78 ± 0.41	11.28 ± 1.08
Octanoic acid	9.39 ± 0.11	4.62 ± 0.6	6.49 ± 0.31	8.56 ± 0.26	8.21 ± 0.34	4.1 ± 0.62	4.64 ± 0.59	5.88 ± 0.14	5.94 ± 0.82
Decanoic acid	2.06 ± 0.15	2.3 ± 0.2	2.35 ± 0.3	1.71 ± 0.18	2.39 ± 0.24	1.56 ± 0.4	2.69 ± 0.48	1.79 ± 0.18	2.31 ± 0.13
ΣMCFA	24.57 ± 0.94	6.92 ± 0.79	8.84 ± 0.07	22.66 ± 0.46	23.97 ± 0.12	12.8 ± 1.11	16.74 ± 0.6	17.45 ± 0.53	19.53 ± 1.76
Dodecanoic acid	n.d.	0.03 ± 0.03	0.06 ± 0.01	n.d.	0.17 ± 0.03	0.14 ± 0.04	0.15 ± 0.1	0.03 ± 0.03	0.14 ± 0.09
Ethyl acetate	7.97 ± 0.31	5.39 ± 0.79	4.36 ± 1.04	8 ± 1.6	7.29 ± 0.5	5.66 ± 0.58	4.88 ± 0.67	6.61 ± 0.64	4.01 ± 0.59
Isobutyl acetate	27.16 ± 1.23	26.67 ± 2.38	23.34 ± 0.78	26.37 ± 2.29	25.62 ± 1.9	25.43 ± 0.69	23.13 ± 0.29	23.83 ± 1.97	18 ± 4.15
Ethyl butanoate	0.55 ± 0.06	0.51 ± 0.1	0.67 ± 0.12	0.48 ± 0.06	0.71 ± 0.16	0.49 ± 0.11	0.72 ± 0.41	1.28 ± 0.27	n.d.
Ethyl hexanoate	1.01 ± 0.4	0.78 ± 0.25	2.08 ± 0.05	1.44 ± 0.11	1.54 ± 0.2	1.2 ± 0.34	1.12 ± 0.14	2.17 ± 0.12	1.44 ± 0.45
Ethyl decanoate	n.d.	0.71 ± 0.35	n.d.	n.d.	n.d.	n.d.	n.d.	1.67 ± 0.36	n.d.
Ethyl dodecanoate	1.53 ± 1.53	0.82 ± 0.82	1.25 ± 1.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ΣEthyl esters	38.22 ± 1.5	34.87 ± 3.06	31.7 ± 1.32	36.29 ± 3.74	35.16 ± 1.98	32.77 ± 1.49	29.86 ± 0.93	35.56 ± 3.19	23.46 ± 4.29
Ethyl lactate	2.49 ± 0.63	2.77 ± 0.06	2.29 ± 0.33	59.85 ± 2.06	28.7 ± 2.47	57.09 ± 5.14	24.22 ± 2.04	43.11 ± 0.99	76.79 ± 3.05
1-propanol	15.97 ± 0.39	35.17 ± 0.9	25.06 ± 3.48	16.84 ± 0.63	17.05 ± 3.01	34.33 ± 2.03	28.03 ± 1.02	26.92 ± 2.54	28.04 ± 4.52
2-methyl-propanol	20.9 ± 2.23	15.93 ± 0.69	16.48 ± 0.01	18.26 ± 0.35	17.9 ± 0.72	13.53 ± 0.2	12.53 ± 1.19	15.07 ± 0.72	16.47 ± 0.54
1-butanol	0.68 ± 0.12	1.07 ± 0.12	0.77 ± 0.12	0.74 ± 0.03	0.86 ± 0.11	1.09 ± 0.16	n.d.	0.75 ± 0.05	1.09 ± 0.15
2- methyl-1-butanol	37.48 ± 1.24	36.53 ± 1.29	36.57 ± 0.37	35.63 ± 0.76	36.1 ± 2.46	31.94 ± 0.86	34.52 ± 0.88	33.45 ± 0.34	36.77 ± 0.55
1-pentanol	0.28 ± 0.08	0.26 ± 0.16	0.5 ± 0.03	0.09 ± 0.16	0.73 ± 0.12	0.46 ± 0.09	0.7 ± 0.11	1.22 ± 0.13	0.91 ± 0.08
cis-3-hexen-1-ol	0.47 ± 0.03	0.77 ± 0	0.85 ± 0.11	0.5 ± 0.01	0.55 ± 0.1	0.79 ± 0.15	0.74 ± 0.02	0.72 ± 0.04	1 ± 0.08
2-butanol	1.1 ± 0	1.05 ± 0.08	0.92 ± 0.21	1.05 ± 0.05	1.56 ± 0.11	1.46 ± 0.04	1.32 ± 0.26	0.89 ± 0.06	1.3 ± 0.33
2-phenylethanol	18.99 ± 0.4	18.07 ± 0.63	19.58 ± 0.62	20.68 ± 0.89	21.31 ± 0.66	18.6 ± 1.19	20.46 ± 0.4	19.96 ± 0.37	22.69 ± 0.77
Phenylmethanol	0.62 ± 0.07	0.72 ± 0.02	0.62 ± 0.08	0.56 ± 0.03	0.96 ± 0.18	0.89 ± 0.07	1.16 ± 0.14	0.55 ± 0.02	1 ± 0.05
ΣFusel alcohols	96.49 ± 3.49	109.57 ± 2.91	101.36 ± 4.3	94.35 ± 2.04	97.03 ± 6.29	103.09 ± 2.93	99.46 ± 3.06	99.52 ± 3.3	109.27 ± 4.17
Isoamilic acetate	3.37 ± 0.41	3.58 ± 0.19	4.9 ± 0.4	3.34 ± 0.27	2.46 ± 0.4	3.35 ± 0.68	3.12 ± 0.27	5.55 ± 0.27	2.72 ± 0.57
Hexyl acetate	0.58 ± 0.03	0.43 ± 0.06	0.59 ± 0.22	n.d.	1.62 ± 0.53	0.53 ± 0.1	1.67 ± 1.43	0.9 ± 0.1	2.59 ± 0.64
2-phenelethyl acetate	0.24 ± 0.04	0.24 ± 0.05	0.47 ± 0.06	0.4 ± 0.09	0.37 ± 0.14	0.33 ± 0.07	0.36 ± 0.1	0.93 ± 0.22	0.44 ± 0.02
ΣSusel alcohol acetates	4.19 ± 0.45	4.25 ± 0.17	5.96 ± 0.65	3.73 ± 0.32	4.44 ± 1.05	4.2 ± 0.84	5.16 ± 1.3	7.38 ± 0.52	5.75 ± 0.06
2.3-butanediol	n.d.	n.d.	n.d.	11.37 ± 1	54.85 ± 11.15	25.13 ± 0.69	76.37 ± 2.6	24.32 ± 2.54	90.49 ± 16.25

CHAPTER III

Effect of yeast lees derived compounds in *Oenococcus oeni* related with non-*Saccharomyces*

In this chapter, we focused on the possible positive effect of some yeast lees derived compounds in MLF. It has been addressed since the past that it exists a positive effect of MLF when it is performed under the presence of yeast lees.

First, we performed MLF with three *O. oeni* strains in presence of simulated yeast lees bellowing to different species: *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* in wine like medium (WLM). The results obtained in this experiment (**Chapter III: 1**) confirmed that the use of different yeast lees species had different impact in MLF. In general, non-*Saccharomyces* had positive, or at least neutral, interactions towards *O. oeni*. From the different parameters studied, the utilization and consumption of mannoproteins caught our attention and was further studied.

Second, we continued working with mannoproteins (**Chapter III: 2**). *O. oeni* can degrade these macromolecules into monosaccharides or smaller peptides that can incorporate. In this sense, we studied the utilization of mannoproteins in WLM with a commercial extract. Besides, we explored the relative expression of some genes related with the uptake and utilization of mannose. We also performed some alcoholic fermentations with some non-*Saccharomyces* as in the previous manuscript. We observed a general utilization of mannoproteins in all cases and an upregulation of those genes related with mannose uptake in response to wine like conditions. Higher consumption of mannoproteins was detected in wines with higher mannoprotein availability, which did not present the highest upregulation. Thus, the degradation seems to be more related with the adaptation to a particular fermenting medium.

CHAPTER III: 1

Simulated lees of different yeast species modify the performance of malolactic fermentation by *Oenococcus oeni* in wine like medium

Aitor Balmaseda[a], Nicolas Rozès[b], Albert Bordons[a],

Cristina Reguant[a,*]

* Corresponding author: cristina.reguant@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

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Abstract

The use of non-*Saccharomyces* yeast together with *S. cerevisiae* in winemaking is a current trend. Apart from the organoleptic modulation of the wine, the composition of the resulting yeast lees is different and may thus impact malolactic fermentation (MLF). Yeasts of Saccharomyces cerevisiae, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* were inactivated and added to a synthetic wine. Three different strains of *Oenococcus oeni* were inoculated and MLF was monitored. The supplementation of lees increased nitrogen compounds but did not always improve MLF. There may be many other compounds regulating these yeast lees-O. oeni interactions apart from the well-known mannoproteins and amino acids. This is the first study of MLF with different *O. oeni* strains in the presence of *S. cerevisiae* and non-*Saccharomyces* yeast lees to report a strain-specific interaction between them.

Keywords

Oenococcus oeni, non-*Saccharomyces*, yeast lees, malolactic fermentation, nitrogen compounds, mannoproteins

Introduction

Malolactic fermentation (MLF) is a biotransformation that occurs in fermented beverages like wine and cider (Davis et al., 1985). This metabolism is a survival adaptation of lactic acid bacteria (LAB) under the stress conditions present in those media, such as low nutrient availability, low pH and high ethanol content. MLF is the decarboxylation of L-malic acid in L-lactic acid with a small increase in pH. It is desirable in those white wines with high acidity and for all red wines in general. Of all the LAB species present in wine, *Oenococcus oeni* is the most important since it is the one that best adapts to the conditions found in wine (Lonvaud-Funel, 1999).

In a spontaneous winemaking process, oenological yeasts through alcoholic fermentation (AF) first ferment the sugars of the grape must. There is a high diversity of yeast species at the beginning of the AF. When the concentration of sugar starts to decrease producing ethanol, that diverse yeast group of non-*Saccharomyces* is rapidly replaced by *S. cerevisiae* (Beltran et al., 2002). As a result of AF, the grape must becomes a poor nutrient medium with low pH and a high concentration of ethanol. Consequently, the yeast-*O. oeni* compatibility is a key factor for successful MLF since this fermentation typically takes places after the AF (Balmaseda et al., 2018). Traditionally, *S. cerevisiae* has been inoculated to ensure a controlled AF, but nowadays there is increasing interest in the possible advantages of using selected non-*Saccharomyces* strains together with *S. cerevisiae* (Padilla et al., 2016). This opens up a new scenario of metabolic activities and chemical modulation in the wines produced, also modifying the media for the subsequent MLF (**Chapter I: 1**).

During AF, LAB from the grape surface or cellar equipment are present in a very low population (González-Arenzana et al., 2012). As time goes by, the yeast lees remaining in the wine begin to lose viability and undergo an autolysis process due to the low metabolic activity and high ethanol concentration. Under these conditions the yeasts lyse and release their intracellular content into the wine (Martínez-Rodriguez et al., 2001), promoting LAB growth (Reguant et al., 2005).

The main changes generally attributed to the lysis of yeasts are the increases in mannoproteins and nitrogen compounds, which are commonly related to a stimulation of MLF performance (Guilloux-Benatier et al., 1995, Alexandre et al., 2004,

Balmaseda et al., 2018). These released macromolecules can be hydrolysed (Manca De Nadra et al., 1999, Folio et al., 2008) by *O. oeni* and assimilated as a nitrogen source, stimulating its metabolism. However, the presence of higher concentrations of yeast extracts is not always linked to a higher protease activity by *O. oeni* (Remize et al., 2006).

The increase in released compounds will depend on the yeast strain. Indeed, some non-*Saccharomyces* such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* are reported to increase mannoprotein concentrations in wine (González-Royo et al., 2015, Ferrando et al., 2020), especially in ageing (Belda et al., 2016). In addition, there are other compounds released during the autolysis process that may have a negative rather than stimulatory effect (Patynowski et al., 2002, Herrero et al., 2003). However, there is little knowledge about the effect of yeast lees on MLF.

In this work we aim to evaluate the MLF performance of some selected *O. oeni* strains in the presence of yeast lees of different species. In particular we consider those compounds in relation to the stimulation of MLF under yeast lees in a defined synthetic wine and the L-malic acid consumption rate and viability of *O. oeni*.

Materials and methods

Experimental fermentations

Fermentations were performed in 250 mL flasks containing 250 mL of sterile wine-like medium (WLM) static at 20 ºC. The WLM was prepared following Bordas et al. (2015), with 12% ethanol (v/v), 2 g/L of L-malic acid and pH 3.4, but with half nitrogen composition than in Bordas et al. (2015): 1.25 g/L of Bacto™ casamino acids (BD, France) and 1.25 g/L of peptone (Panreac, Química SLU, Castellar del Vallès, Spain). Yeasts for supplementation of WLM in the form of simulated yeast lees were grown in sterile concentrated must (65.4 ° Brix; Mostos Españoles S.A., Tomelloso, Spain) diluted to a concentration of 200 g/L of glucose and fructose (Martín-García et al., 2020). Seven yeasts belonging to the species *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* of different origins were used (Table 13). After 2 weeks' incubation, the yeast population was counted in a Neubauer chamber. An appropriate volume of the

fermenting must was centrifuged (8,500 rpm, 10') to achieve a final concentration of 10^7 CFU/mL in 2 L of WLM, which corresponded to an average biomass of 1.9 ± 0.39 mg/mL (wet weight) in the synthetic medium. The resulting pellet was resuspended in 50 mL of WLM. At this point the collected yeast biomass was inactivated by heating in three cycles of 1 min at 90 ºC with 1 min in ice bath between each. The cells were then disrupted using a One Shot disruptor (Constant Systems Ltd., United Kingdom) at 5 ºC, applying 2.5 kbar pressure (Margalef-Català et al., 2016). The aim of the disruption process was to simulate the yeast cell status in the final stages of AF, when some of them are already lysed but others are still intact with low viability (<10^3 CFU/mL). YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac) was used to calculate the total number of viable yeast cells after incubation at 28 °C for 48 h.

Table 13. Microorganisms used in this work.

Abbreviation	Species	Strain name	Source
ScQA23	*S. cerevisiae*	Lalvin-QA23	Lallemand S.L.
Sc3D	*S. cerevisiae*	Viniferm-3D	Agrovin S.A.
TdBiodiva	*T. delbrueckii*	Biodiva	Lallemand S.L.
TdViniferm	*T. delbrueckii*	Viniferm NS-TD	Agrovin S.A.
TdTDP	*T. delbrueckii*	CECT 13135	BE-URV[1]
MpFlavia	*M. pulcherrima*	Flavia	Lallemand S.L.
MpMPP	*M. pulcherrima*	CECT 13131	BE-URV[1]
PSU-1	*O. oeni*	ATCC BAA-331	ATCC*
CH11	*O. oeni*	Viniflora-CH11	Chr. Hansen S.L.
1Pw13	*O. oeni*	CECT 8893	BE-URV[2]

BE-URV: Biotecnologia Enològica research group at the Universitat *Rovira i Virgili*, Tarragona, Catalonia, Spain.

[1] From Padilla et al., 2016; [2] From Franquès et al., 2017

*ATCC: American Type Culture Collection

Each WLM condition (250 mL), including the yeast lees, was then inoculated with one of the three *O. oeni* strains (Table 13) for a population of around 2 x 10^7 CFU/mL. All the strains were pre-cultured in MRSmf broth at 28ºC for three days (Margalef-Català et al., 2017) before inoculation in each WLM. These fermentations were carried

out in triplicate at 20 °C. Samples were taken every 24 h to monitor the evolution of L-malic acid consumption and the bacterial population. Samples were plated on MRSmf (Margalef-Català et al., 2017) and incubated at 27 °C in a 10% CO_2 atmosphere for 7 days. MLF was considered to have finished when the L-malic acid was below 0.1 g/L.

Synthetic wines characterisation

The synthetic wines were characterised after supplementation with the yeast lees (initial: t_0 MLF) and after MLF completion of each *O. oeni* strain. Samples were centrifugated (8,500 rpm, 10') and kept frozen at -20 ºC until analysis. pH was measured before freezing (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain) and various compounds (acetic acid, citric acid, L-lactic acid, L-malic acid, succinic acid and glucose + fructose) were analysed using a Miura One multianalyser (TDI SL, Gavà, Spain).

The total soluble protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) (Sigma150 Aldrich, St Louis, MO, USA) and dye reagents (Bio-Rad, Hercules, CA, USA) for the calibration curve.

The mannoprotein content of the WLM before and after MLF was quantified using a D-mannose and D-glucose assay kit K-MANGL (Megazyme, Wicklow, Ireland). Briefly, 25 mL of 95% (v/v) ethanol was added to 5 mL of each sample, vortexed and precipitated overnight at 4 ºC. Each pellet obtained was washed twice with 10 mL of 95% ethanol and centrifugated (4,500 rpm, 10'). The pellets were then transferred to 2 mL tubes and dried at 30 ºC for 30' in vacuum (Concentrator Plus, Eppendorf, Hamburg, Germany). Afterwards they were resuspended in 1 mL of 5 M H_2SO_4, incubated at 90 ºC for 1h and neutralised with 1mL of 10 M NaOH. Finally, the sample was centrifugated (8,500 rpm, 5') and the supernatant kept for analysis. The free sugar (D-glucose, D-fructose and D-mannose) content was then determined in accordance with the manufacturer's instructions.

Amino acid composition analysis

The amino acid and ammonium content was analysed by HPLC following the method described by Gómez-Alonso et al. (2007). 400 µL of sample was filtered

through 0.22 µm syringe filter into a vial. Afterwards, 700 µL of 1M borate buffer at pH 9 (adjusted with NaOH 10N), 300 µL of methanol, 10 µL of internal standard (L-2-aminoadipic acid, 1 g/L) and 15 µL of DEEM (diethyl ethoxymethylenemalonate, Fluka, Germany) were added. The vial was encapsulated and vortexed for 30 seconds. The samples were then derivatised for 2 h at 80 °C. After that, 50 µL of each sample was directly injected into the HPLC. The HPLC (Agilent 1100, Agilent Technologies, Germany) was equipped with a DAD ultraviolet (Agilent Technologies, Germany), and separation was performed on a Hypersil ODS C18 column (Agilent Technologies, Germany) with a particle size of 5 µm (250 mm x 4.6 mm) and thermostated at 20 °C. The mobile phase (Buffer A) consisted of 2.05 g/L of sodium acetate anhydrous and 0.2 g/L of sodium azide with MilliQ water (Millipore Q-PODTM Advantage A10) adjusted to pH 5.8 with glacial acetic acid, while the mobile phase (Buffer B) consisted of 80% (v/v) acetonitrile and 20% (v/v) methanol (Panreac, Spain). The concentration of each amino acid was calculated with an external standard curve and the signal of each sample was normalised with the area of the internal standard. The amino acid and ammonium content was transformed into yeast assimilable nitrogen (YAN, expressed as mg N/L) depending on the proportion of nitrogen atoms of each amino acid.

Statistical analysis

The statistical software used was XLSTAT version 2020.2.3 (Addinsoft, Paris, France). The data obtained underwent a two-way ANOVA (yeast lees and *O. oeni* strain as qualitative variables) with a subsequent analysis using the Tukey test, with a confidence interval of 95% and significant results with a p-value ≤ 0.05. Optimization indexes (OI) for MLF performance were calculated based on Borrull et al. (2016) for each *O. oeni* strain in the 8 synthetic wines. The variables used for this calculation were MLF duration, MLF rate, pH, mannose content, ammonium content and total amino acid content. Values were normalised using the highest value for each parameter as (x/reference value). In the case of MLF duration, where the highest value represents the worst performance, the calculation was 1-(x/reference value). Principal component analysis (PCA) was performed with the obtained OI using the same statistical software.

Results and discussion

Fermentations

The duration of MLF in the control condition (WLM) was the same with *O. oeni* 1Pw13 and CH11 (11 days) whereas with *O. oeni* PSU-1 it was 4 days shorter (Table 14, Suppl. Figure S6). The addition of yeast lees influenced MLF duration depending on the yeast strain used to obtain the lees and the *O. oeni* strain inoculated. In most of the fermentations with PSU-1 and 1Pw13 strains slight delays were observed in MLF with respect to the control condition (WLM). However, the addition of Sc3D lees with PSU-1 and 1Pw13, and QA23 lees with 1Pw13, caused a remarkable slowdown in MLF. Treatments with WLM-Td Biodiva were the only ones showing a shorter MLF with PSU-1 and 1Pw13 with respect to the control WLM. Instead, CH11 showed a completely different behaviour compared to the other *O. oeni* strains. Most of the fermentations of CH11 with lees were shorter than the control condition, except in WLM-TdTDP, that showed no statistical differences (Table 14, Suppl. Figure S6). The performance in WLM-Sc3D clearly illustrates the differences among *O. oeni* strains. In WLM-Sc3D, the MLF with PSU-1 was stuck at around 0.3 g/L of L-malic acid and with 1Pw13 MLF took 30 days. Meanwhile, CH11 showed the fastest MLF among all conditions (4.3 days) with Sc3D lees (Table 14, Suppl. Figure S6). Globally, *O. oeni* 1Pw13 was the slowest fermenting strain of the three, with the exception of its performance in WLM-TdBiodiva and WLM-TdTDP, whereas the most positively affected strain by supplementation with yeast lees was *O. oeni* CH11.

Regarding the yeast strains, the strongest negative impact on MLF duration observed was associated to *S. cerevisiae* lees addition, mainly with Sc3D, whereas treatments with *T. delbrueckii* Biodiva lees were the only ones causing a shortening of MLF with respect to the control condition in the three *O. oeni* strains studied, being significant in two of them.

The differences in MLF duration correlated well, in most of the cases, with the differences observed in L-malic acid consumption rate, being CH11 the strain showing the highest rates and 1Pw13 the strain with the lowest rates (Table 14). *O. oeni* PSU-1

showed, in general, intermediate L-malic acid consumption rates when compared to the other two strains.

Table 14. Malolactic fermentation (MLF) duration and the consumption rate of the three *O. oeni* strains (PSU-1, 1Pw13 and CH11) in the different wine-like media (WLM) with yeast lees. Values shown are the mean of triplicates ± SD. The duration of *O. oeni* PSU-1 in WLM-Sc3D was excluded from the analysis as that MLF did not finish.

	Duration (days)			Consumption rate (L-malic acid g/L·d)*		
	PSU-1	1Pw13	CH11	PSU-1	1Pw13	CH11
WLM	7 ± 0.0^{c}	11 ± 0.0^{e}	11 ± 0.0^{a}	0.35 ± 0.01^{deC}	0.24 ± 0.00^{bA}	0.30 ± 0.01^{eB}
WLM-ScQA23	6.5 ± 0.5^{cdA}	17.5 ± 0.5^{bC}	8.7 ± 0.6^{bB}	0.49 ± 0.00^{bC}	0.14 ± 0.00^{deA}	0.40 ± 0.01^{dB}
WLM-Sc3D	-	30 ± 0.0^{aB}	4.3 ± 0.6^{dA}	0.16 ± 0.00^{gB}	0.12 ± 0.01^{eA}	0.62 ± 0.06^{aC}
WLM-TdBiodiva	5.7 ± 0.6^{dA}	8 ± 0.0^{fB}	10.8 ± 0.3^{aC}	0.52 ± 0.02^{aB}	0.29 ± 0.01^{aA}	0.55 ± 0.00^{bcB}
WLM-TdViniferm	9 ± 0.0^{b}	12 ± 0.0^{d}	5 ± 0.0^{cd}	0.44 ± 0.01^{cB}	0.2 ± 0.01^{cA}	0.49 ± 0.01^{cC}
WLM-TdTDP	8.7 ± 0.6^{bA}	11.3 ± 0.6^{deB}	11.7 ± 0.6^{aB}	0.27 ± 0.00^{fB}	0.15 ± 0.00^{dA}	0.39 ± 0.01^{dC}
WLM-MpFlavia	11 ± 0.0^{aB}	12.8 ± 0.3^{cC}	8.7 ± 0.6^{bA}	0.34 ± 0.01^{eB}	0.19 ± 0.01^{cA}	0.35 ± 0.03^{deB}
WLM-MpMPP	9 ± 0.0^{bB}	12 ± 0.0^{dC}	5.7 ± 0.6^{cA}	0.36 ± 0.02^{dB}	0.14 ± 0.02^{deA}	0.57 ± 0.00^{abC}

$^{a–g}$ Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences among the values of the same O. oeni strain in the different synthetic wines. Uppercase letters correspond to differences between values of the three strains in the same synthetic wine after MLF. The absence of uppercase letter in the duration of some synthetic wines is due to the lack of SD. * Consumption rate of L-malic acid was calculated considering the period of exponential decrease of this acid.

As mentioned earlier, the yeast lees viability was low, at around 500 CFU/mL, and decreased during MLF to less than 100 CFU/mL in some cases (data not shown). Presumably, therefore, there was no inhibition due to viable yeast metabolic activity.

The presence of yeast lees during MLF can modulate MLF performance. The yeast metabolites released during autolysis can have a stimulatory or a negative effect on MLF depending on the *O. oeni* fermenting strain (Patynowski et al., 2002, Herrero et al., 2003). Patynowski et al., (2012) reported a longer lag phase of *O. oeni* growth in wines with longer contact with yeast lees. Also, supplementation with commercial yeast extracts in the MLF of cider is described as being possibly more stimulatory to *O. oeni* than supplementation with recovered yeast lees (Herrero et al., 2003). In the present work we confirm that the effect of yeast lees on MLF performance strongly depends on the fermenting *O. oeni* strain and on the yeast lees strain.

The different patterns observed in these MLF performances could be related to the specific nutritional requirements of each *O. oeni* strain and the compatibility between each yeast-*O. oeni* strain couple.

Viability

The changes observed in *O. oeni* viability at the end of MLF were variable depending on the added lees and the inoculated strain (Figure 20). *O. oeni* PSU-1 lost around one logarithmic unit in most of the synthetic wines, with the exception of the control WLM and WLM-TdViniferm, where the viability was maintained. In WLM-Sc3D, *O. oeni* PSU-1 suffered a drastic decrease in viability that led to the unfinished MLF. On the other hand, a slight decrease was observed in viability of strains 1Pw13 and CH11 in most of the conditions. These two strains also showed some exceptions in which the viability was maintained or slightly increased: WLM-TdViniferm and WLM-TdTDP, for 1Pw13, and WLM-TdBiodiva and WLM-MpMPP, for CH11.

The observed variation in viability was not directly related to MLF duration or consumption rates (Table 14), with the exception of *O. oeni* PSU-1 in the WLM-Sc3D wine. In general, the bacterial population at the end of MLF was enough (Reguant et al., 2005), i.e. higher than 10^6 CFU/mL, to ensure MLF performance (Table 14, Figure 20). Consequently, the effect of yeast lees on MLF, excluding Sc3D, may be associated with an inhibition of MLF capacity and not with a loss of viability.

Chemical parameters

The supplementation with yeast lees changed significantly some parameters of WLM (Table 15). Some significant changes were observed in pH, such as in WLM-TdBiodiva and WLM-TdTDP, which increased by 0.18 and 0.22 pH units, respectively, compared to control. As the wine-like conditions represent a highly acidic environment in which *O. oeni* has to grow, any increase in that value may improve membrane integrity and cell survival (Tourdot-Maréchal et al., 2000). Higher initial pH values may be one of the stimulating factors regarding the duration of MLF in the case of WLM-TdBiodiva, but not the only one, since this is not observed with all strains of *O. oeni*. As a result of the increased initial pH value, the values of those synthetic wines after MLF were the highest (Table 15).

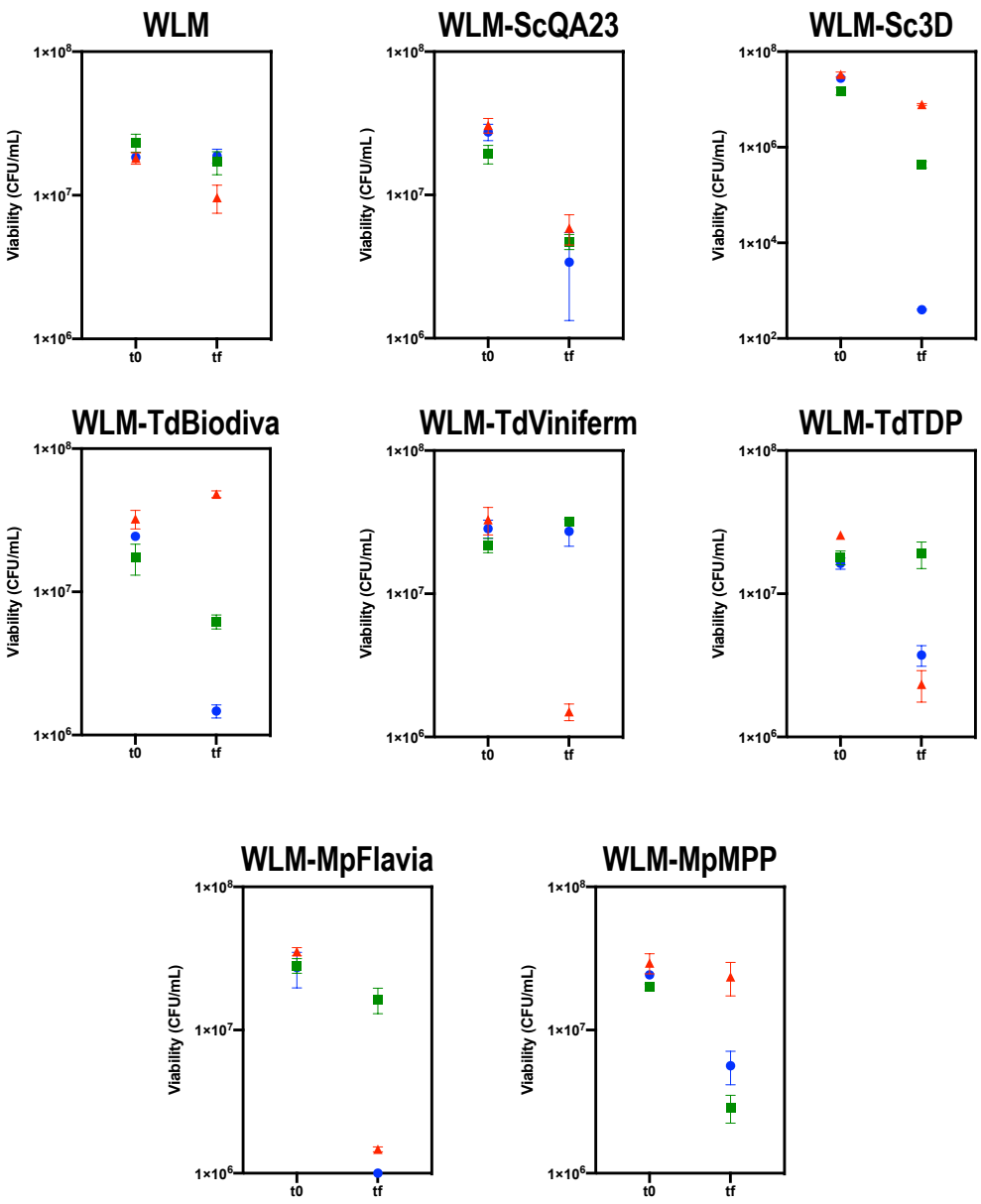


Figure 20. Viability of the three studied *O. oeni* strains in the different wine-like media (WLM) at the beginning of MLF (t_0) and after MLF, when [L-malic acid] < 0.1 g/L (t_f). (●) *O. oeni* PSU-1, (■) *O. oeni* 1Pw13, (▲) *O. oeni* CH11.

In most of the cases, sugars were not consumed by *O. oeni* during MLF. However, a significant decrease in sugars was observed at the end of MLF with PSU-1 and 1Pw13 in WLM-Sc3D and WLM-MpFlavia, and also with 1Pw13 in WLM-ScQA23 (Table 3). No increase in acetic acid was observed in all these assays (Table 3), which could be linked to sugar consumption by *O. oeni*. Moreover, these MLF were slower than the rest of the fermentations for these two *O. oeni* strains. Altogether, it may be possible that the remaining viable yeast in the lees were responsible for sugar consumption in those MLF that *O. oeni* had a weak malolactic activity.

Table 15. Oenological parameters of wine-like media (WLM) before *O. oeni* inoculation (Initial, after yeast lees addition in supplemented WLM) and after malolactic fermentation (MLF) of the three *O. oeni* strains (PSU-1, 1Pw13 and CH11). Values shown are the means of triplicates ± SD.

		Glucose+ fructose (g/L)	Citric acid (g/L)	Acetic acid (g/L)	pH	Proteins (mg/mL)	Amino acids (mg N/L)	NH_4 (mg/L)
WLM	Initial	2.12±0.00^{a}	0.48±0.00^{a}	0.20±0.00^{b}	3.40±0.00^{e}	0.28±0.00^{e}	123.70±0.00^{f}	8.73±0.00^{de}
	PSU-1	1.99 ± 0.07^{aA}	0.20 ± 0.01^{aB}	0.31 ± 0.01^{bcA}	3.69 ± 0.02^{cA}	0.21 ± 0.01^{cA}	117.86 ± 8.64^{bcA}	4.45 ± 1.11^{bA}
	1Pw13	2.01 ± 0.02^{aA}	0.29 ± 0.00^{abA}	0.28 ± 0.01^{abB}	3.65 ± 0.01^{dA}	0.24 ± 0.02^{abA}	117.13 ± 6.04^{bcA}	5.75 ± 0.08^{bcA}
	CH11	1.99 ± 0.02^{bcA}	0.20 ± 0.03^{abB}	0.33 ± 0.01^{bcA}	3.65 ± 0.04^{dA}	0.23 ± 0.01^{cA}	116.07 ± 3.95^{cA}	4.92 ± 0.25^{cA}
WLM-ScQA23	Initial	2.11 ± 0.00^{a}	0.44 ± 0.00^{b}	0.20 ± 0.00^{b}	3.35 ± 0.00^{g}	0.26 ± 0.00^{f}	142.39 ± 0.00^{c}	8.04 ± 0.00^{e}
	PSU-1	1.98 ± 0.05^{aA}	0.10 ± 0.03^{dB}	0.32 ± 0.02^{bA}	3.60 ± 0.02^{dA}	0.26 ± 0.01^{bcA}	107.65 ± 15.36^{bcAB}	4.33 ± 0.09^{bB}
	1Pw13	n.d.^{cB}	0.18 ± 0.03^{cA}	0.25 ± 0.05^{bA}	3.55 ± 0.01^{eB}	0.24 ± 0.04^{abA}	87.71 ± 3.11^{eB}	0.52 ± 0.17^{dC}
	CH11	2.00 ± 0.02^{bcA}	0.16 ± 0.01^{abAB}	0.32 ± 0.01^{bcdA}	3.60 ± 0.01^{eA}	0.22 ± 0.02^{cA}	118.05 ± 6.91^{cA}	6.14 ± 0.11^{cA}
WLM-Sc3D	Initial	2.00 ± 0.00^{d}	0.48 ± 0.00^{ab}	0.20 ± 0.00^{b}	3.45 ± 0.00^{c}	0.34 ± 0.01^{a}	142.88 ± 0.00^{c}	9.02 ± 0.00^{cd}
	PSU-1	0.10 ± 0.04^{cB}	0.17 ± 0.01^{abc}	0.27 ± 0.01^{dB}	3.62 ± 0.02^{dC}	0.26 ± 0.02^{bcA}	98.74 ± 1.58^{cAB}	n.d.^{cC}
	1Pw13	n.d.^{cB}	0.18 ± 0.04^{cA}	0.28 ± 0.01^{abB}	3.67 ± 0.01^{cdB}	0.23 ± 0.01^{bB}	104.56 ± 4.89^{cdA}	1.34 ± 0.25^{dB}
	CH11	1.96 ± 0.04^{cAA}	0.08 ± 0.00^{cB}	0.37 ± 0.01^{aA}	3.76 ± 0.01^{bA}	0.24 ± 0.01^{bcAB}	93.95 ± 2.70^{dB}	4.59 ± 0.30^{cA}
WLM-TdBiodiva	Initial	2.00 ± 0.00^{d}	0.48 ± 0.00^{ab}	0.21 ± 0.00^{b}	3.58 ± 0.00^{b}	0.29 ± 0.00^{d}	152.62 ± 0.00^{b}	10.00 ± 0.00^{bc}
	PSU-1	2.11 ± 0.02^{aA}	0.18 ± 0.01^{abC}	0.31 ± 0.01^{bcA}	3.92 ± 0.02^{bA}	0.38 ± 0.07^{aA}	118.76 ± 5.53^{bcA}	5.92 ± 0.68^{abAB}
	1Pw13	2.00 ± 0.04^{aB}	0.30 ± 0.00^{aA}	0.27 ± 0.02^{abB}	3.87 ± 0.01^{bB}	0.30 ± 0.06^{abA}	114.68 ± 8.90^{bcA}	4.59 ± 0.47^{cB}
	CH11	2.06 ± 0.02^{abAB}	0.2 ± 0.01^{aB}	0.3 ± 0.01^{cdeA}	3.9 ± 0.01^{aAB}	0.32 ± 0.01^{aA}	122.2 ± 6.28^{bcA}	7.67 ± 1.26^{cA}
WLM-TdViniferm	Initial	2.09 ± 0.00^{b}	0.50 ± 0.00^{a}	0.21 ± 0.00^{b}	3.42 ± 0.00^{d}	0.30 ± 0.02^{c}	134.39 ± 0.00^{d}	8.87 ± 0.00^{de}
	PSU-1	2.05 ± 0.03^{aA}	0.13 ± 0.00^{cdC}	0.30 ± 0.02^{bcdA}	3.71 ± 0.01^{cA}	0.31 ± 0.05^{abA}	126.01 ± 4.59^{bB}	7.20 ± 1.04^{aB}
	1Pw13	2.03 ± 0.03^{aAB}	0.24 ± 0.01^{bA}	0.25 ± 0.00^{bB}	3.68 ± 0.00^{cB}	0.33 ± 0.04^{aA}	129.62 ± 2.55^{bAB}	10.75 ± 0.18^{aA}
	CH11	1.96 ± 0.02^{cB}	0.19 ± 0.02^{abB}	0.27 ± 0.02^{eAB}	3.71 ± 0.01^{cA}	0.30 ± 0.02^{aA}	135.91 ± 1.45^{abA}	11.78 ± 0.42^{bA}
WLM-TdTDP	Initial	2.09 ± 0.00^{b}	0.50 ± 0.00^{a}	0.21 ± 0.00^{b}	3.62 ± 0.00^{a}	0.26 ± 0.01^{f}	124.45 ± 0.00^{e}	33.82 ± 0.00^{a}
	PSU-1	2.08 ± 0.07^{aA}	0.15 ± 0.00^{bcC}	0.31 ± 0.01^{bcA}	3.98 ± 0.02^{aA}	0.29 ± 0.03^{abcA}	153.59 ± 12.06^{aA}	n.d.^{cB}
	1Pw13	2.05 ± 0.07^{aA}	0.24 ± 0.01^{bA}	0.27 ± 0.01^{abB}	3.94 ± 0.01^{aB}	0.28 ± 0.00^{abA}	156.66 ± 8.71^{aA}	n.d.^{dB}
	CH11	2.07 ± 0.05^{abA}	0.19 ± 0.01^{abB}	0.29 ± 0.02^{deAB}	3.92 ± 0.02^{aB}	0.31 ± 0.03^{aA}	152.3 ± 7.42^{aA}	26.28 ± 3.13^{aA}
WLM-MpFlavia	Initial	2.09 ± 0.00^{b}	0.48 ± 0.00^{ab}	0.21 ± 0.00^{b}	3.33 ± 0.00^{h}	0.32 ± 0.01^{b}	162.22 ± 0.00^{a}	10.26 ± 0.00^{b}
	PSU-1	0.76 ± 0.47^{bB}	0.20 ± 0.03^{aAB}	0.28 ± 0.01^{cdB}	3.54 ± 0.01^{eB}	0.26 ± 0.03^{bcA}	95.92 ± 3.12^{cB}	n.d.^{cB}
	1Pw13	0.88 ± 0.54^{bB}	0.25 ± 0.00^{abA}	0.27 ± 0.01^{abB}	3.55 ± 0.01^{eB}	0.26 ± 0.02^{abA}	93.07 ± 4.01^{deB}	0.81 ± 0.45^{dB}
	CH11	1.96 ± 0.05^{cA}	0.16 ± 0.03^{bB}	0.32 ± 0.01^{bcdA}	3.59 ± 0.01^{eA}	0.28 ± 0.02^{abA}	117.89 ± 8.88^{cA}	7.88 ± 1.31^{cA}
WLM-MpMPP	Initial	2.06 ± 0.00^{c}	0.45 ± 0.00^{b}	0.23 ± 0.00^{a}	3.38 ± 0.00^{f}	0.28 ± 0.00^{e}	122 ± 0.00^{g}	33 ± 0.00^{a}
	PSU-1	2.06 ± 0.01^{aA}	0.16 ± 0.01^{abcB}	0.37 ± 0.01^{aA}	3.62 ± 0.00^{dA}	0.27 ± 0.02^{bcA}	125.49 ± 4.17^{bA}	6.73 ± 0.02^{aA}
	1Pw13	2.06 ± 0.01^{aA}	0.29 ± 0.02^{abA}	0.31 ± 0.02^{aB}	3.68 ± 0.00^{cA}	0.26 ± 0.03^{abA}	114.37 ± 5.89^{bcA}	6.32 ± 1.19^{bA}
	CH11	2.14 ± 0.05^{aA}	0.2 ± 0.01^{abB}	0.35 ± 0.01^{abA}	3.68 ± 0.00^{cdA}	0.28 ± 0.01^{abA}	112.85 ± 6.65^{cA}	5.67 ± 1.01^{cA}

$^{a–h}$ Values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences among the values of the same *O. oeni* strain in the different synthetic wines. Lowercase

letters also apply to the comparison among the values of the initial synthetic wines. Uppercase letters correspond to differences between values of the three strains in the same synthetic wine after MLF. n.d.: not detected.

Under stress conditions, *O. oeni* can consume citric acid (Davis et al., 1986). In this study, the initial citric acid was consumed approximately between 40 and 80% at the end of the MLF depending on the fermentation (Table 15). In general, the *O. oeni* 1Pw13 was the lowest consumer of this organic acid. WLM-Sc3D was the only synthetic wine in which all three strains consumed more citric acid (less than 0.2 g/L was quantified after MLF). In all cases, acetic acid increased after MLF as a consequence of citric acid consumption (Table 15). In addition, succinic acid, a possible MLF inhibitor (Balmaseda et al., 2018), was also analysed but not detected in the synthetic wines (data not shown).

As for the substrate and product of the MLF, the concentration of each was homogeneous in all the synthetic wines with the exception of those stuck fermentations in which remaining traces of L-malic acid were detected (Suppl. Figure S6) and less L-lactic acid was quantified (data not shown).

One of the metabolisms of interest in the fermentations tested was the assimilation of mannoproteins. As a result of yeast lees supplementation, increased concentrations of mannose (mannoproteins) were quantified in the initial WLM (Figure 21). Some studies have reported a higher mannoprotein concentration in aged wines with non-*Saccharomyces* yeast as regards *S. cerevisiae*, specially *T. delbrueckii* and *M. pulcherrima* (González-Royo et al., 2015, Belda et al., 2016, Benito 2018, Benito et al., 2019). In this study, the highest concentrations of mannoproteins were observed in two of the three tested *T. delbrueckii,* Biodiva and Viniferm, whereas *M. pulcherrima* strains showed lower concentrations than *S. cerevisiae* strains (Figure 21). Surprisingly, although *S. cerevisiae* Viniferm-3D is described as an overproducer of mannoproteins (Belda et al., 2016), under our fermentation conditions it only produced 80 mg eq. mannose/L.

Under oenological conditions *O. oeni* can hydrolyse mannoproteins, and the released products (Jamal et al., 2013), e.g. mannose, can be assimilated as a carbon source by the bacterium (Cibrario et al., 2016). The use of mannose depended on the *O. oeni* strain and the medium it was fermenting (Figure 21). In most of the cases, *O.*

oeni PSU-1 and CH11 showed high consumption of this monosaccharide whereas *O. oeni* 1Pw13 showed lower assimilation of mannose. Indeed, no consumption of mannose was observed when *O. oeni* 1Pw13 fermented in WLM-MpMPP. In contrast, all three strains consumed nearly all the mannose content of WLM-Sc3D.

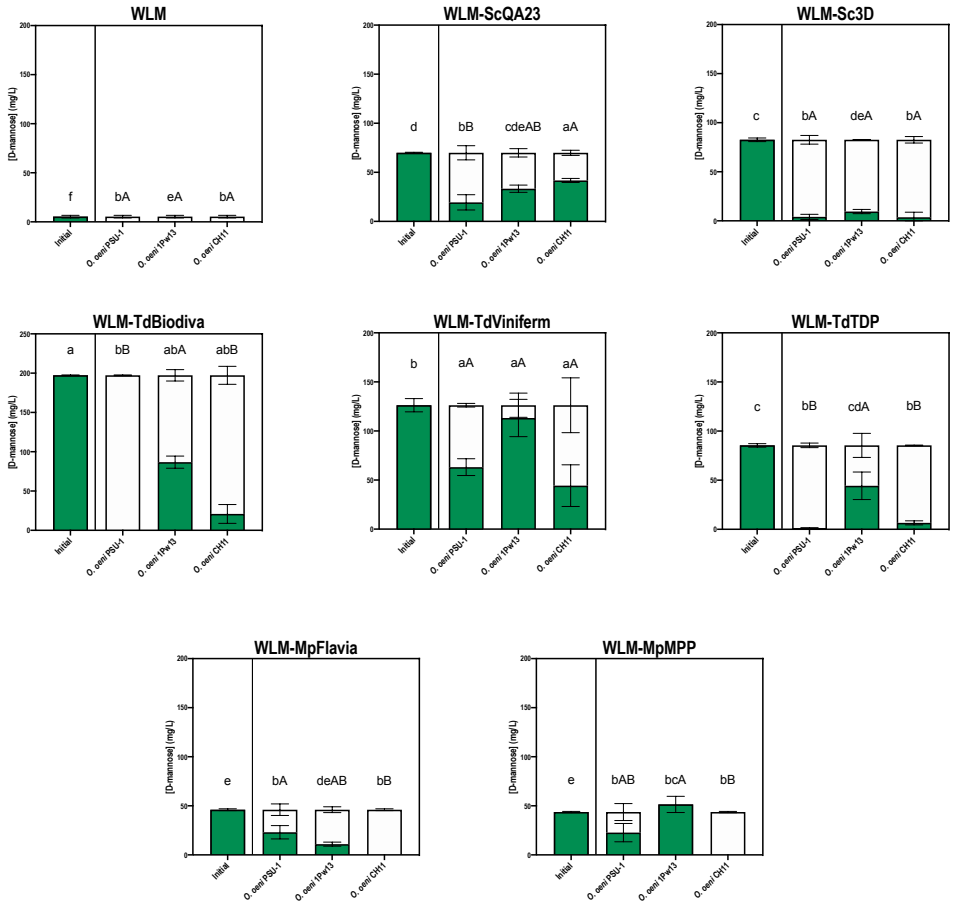


Figure 21. D-mannose concentration detected in wine-like media (WLM) at the beginning of MLF (Initial) and after MLF for *O. oeni* PSU-1, 1Pw13 and CH11. Histograms reflect the consumption of D-mannose during MLF by *O. oeni* as (■) detected D-mannose and (□) consumed D-mannose as the difference between the initial concentration and the concentration detected after MLF. Values shown are the means of triplicates ± SD. Lowercase letters correspond to differences among the values of the same *O. oeni* strain in the different synthetic wines. Lowercase letters also apply to the comparison among the values of the initial synthetic wines. Uppercase letters correspond to differences between values of the three strains in the same synthetic wine after MLF.

It seems that mannoprotein consumption may be strain specific with complex regulation (Cibrario et al., 2016), since each strain behaves quite differently in the different wines and not always proportionally to the mannoprotein concentration (Remize et al., 2005). In view of our results, we could not relate a higher consumption of mannoproteins to a quicker MLF. Nevertheless, we did observe that *O. oeni* 1Pw13, which presented the lowest mannoprotein consumption, had the worst MLF

performance of the strains tested. Moreover, the highest initial concentration of mannoproteins, as well as the highest mannose consumption, was detected in WLM-TdBiodiva, where the MLF were faster for all the *O. oeni* strains with respect to the control (WLM). Considering altogether, the ability of mannoproteins utilization may play a role in the stress response of *O. oeni* in oenological conditions.

Protein and amino acid content

Supplementation with yeast lees can increase the soluble proteins in WLM due to the autolytic process (Martínez-Rodriguez et al., 2001). Four out of the seven synthetic wines with added lees showed a significant increase of protein concentration (Table 15), the highest being those of *S. cerevisiae* 3D, *T. delbrueckii* Viniferm and *M. pulcherrima* Flavia.

During MLF, protein concentrations can increase as a consequence of the progress of yeast autolysis or decrease as *O. oeni* hydrolyses proteins to smaller peptides or amino acids (Manca De Nadra et al., 1999, Folio et al., 2008). Consequently, the variation in the quantification of proteins (Table 15) and amino acids (Table 15, Figure 22) is the sum of (i) the protein release from autolytic yeast lees, except for the control WLM, (ii) the hydrolysis of proteins and release of amino acids, and (iii) the assimilation of amino acids by *O. oeni*. In the synthetic wines with *S. cerevisiae* and *M. pulcherrima* lees, the protein concentration decreased slightly during MLF, as well as in the control WLM. However, a mild increase in protein concentration was observed in the synthetic wines with *T. delbrueckii* lees at the end of MLF (Table 15). In general, these changes were the same whatever the *O. oeni* strain inoculated.

The addition of yeast lees increased the amino acid concentration in most of the cases, with the exception of WLM-MpMPP (Table 15, Figure 22A). After MLF, there was a decrease of the amino acid concentration with the exception of WLM-TdTDP, in which the amino acid concentration increased (Table 15). We could not correlate the higher amino acid release in WLM-TdTDP during MLF to the decrease in protein concentration. A lower consumption of amino acids by *O. oeni* in these fermentations, or a higher peptidase activity, may have been the cause of the higher concentration of free amino acids with respect to the rest of the conditions.

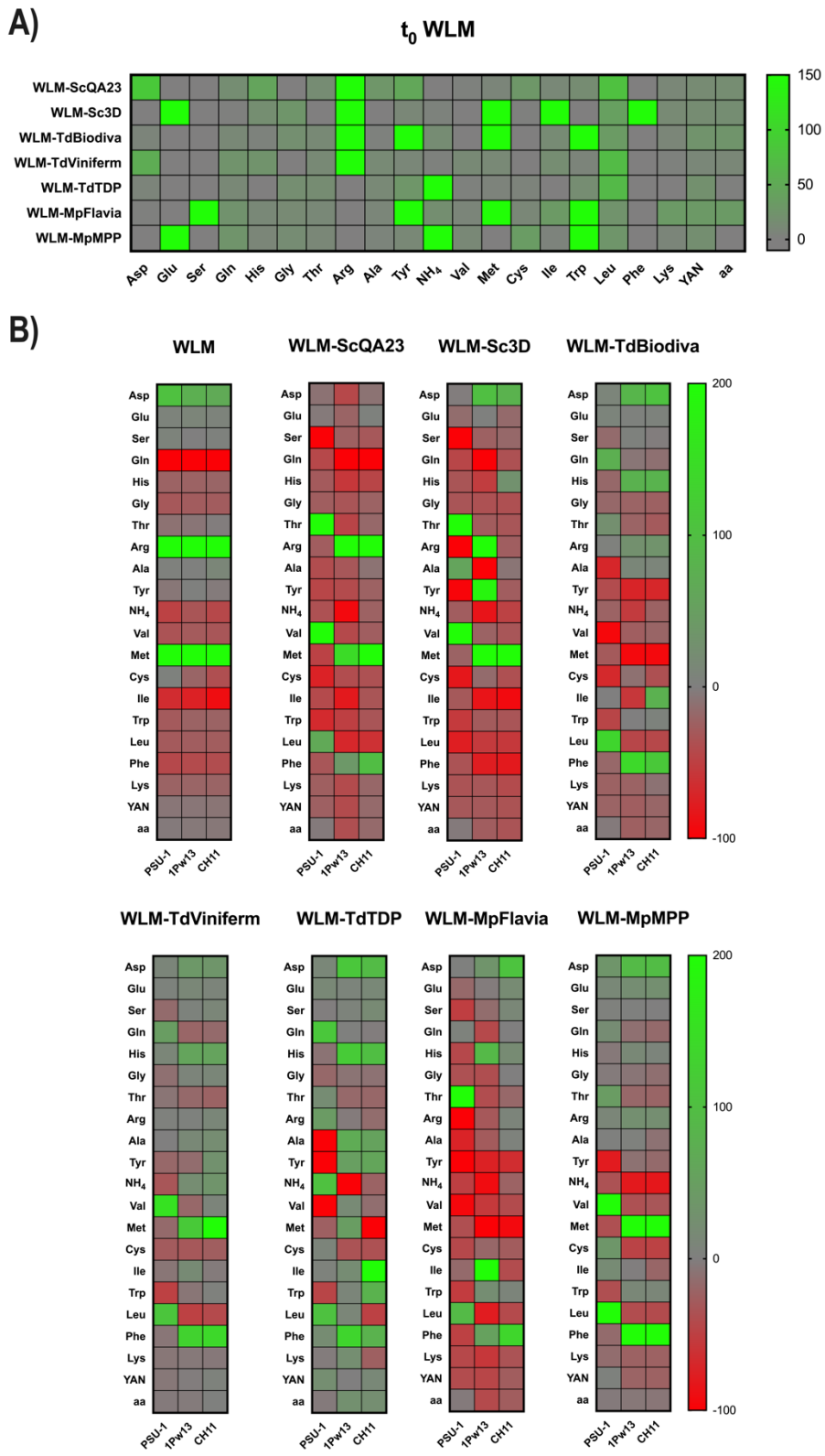


Figure 22. Amino acid analysis of the wine-like media (WLM). A) Enrichment of amino acids in each WLM supplemented with the yeast lees expressed as a percentage of the increase at the beginning (t_0) of malolactic fermentation (MLF). B) Variation in amino acid concentration as a result of MLF represented as a percentage of the increase (green) or decrease (red). No variation (black) is used as the baseline. YAN and total amino acid are also represented in these figures (A and B).

The increase in YAN concentration before MLF ranged in mean value from 8.5% (WLM-TdViniferm) to 30% (WLM-MpFlavia). In most of the cases, the increase was due to certain amino acids and not to an increase in ammonium concentration. Arginine and tryptophan were the most increased amino acids (Figure 22A). Nevertheless, the TdTDP and MpMPP strains significantly increased the ammonium concentration with respect to the control condition without yeast lees addition (Table 15).

As far as the variation in YAN composition during MLF is concerned, some compounds decreased as *O. oeni* assimilated them, while others increased (Figure 22B). As observed in protein concentration, it is difficult to assess the assimilation patterns of each amino acid since the extracellular quantification is the sum of the assimilation of amino acids and the hydrolysis of macromolecules. There is no general agreement on amino acid metabolism for *O. oeni* during MLF. According to the available literature, its patterns are strain specific and, as observed in the present study (Figure 22), also depend on the media. However, some amino acids such as asparagine and histidine seem to undergo less change after MLF (Pozo-Bayón et al., 2005).

In the control condition (WLM), all three *O. oeni* strains had similar patterns of amino acid metabolism. The most consumed amino acids were glutamine and isoleucine, whereas asparagine, arginine and methionine increased their concentrations (Figure 22B). These latter amino acids were probably released due to protease activity and not assimilated by *O. oeni* while the other released products were incorporated. The preference of amino acids in *O. oeni* has not been as widely studied as it has been in wine yeasts. Nevertheless, the consumed glutamine and usually the isoleucine are classified as good nitrogen sources for yeast (Roca-Mesa et al., 2020), and this could explain their consumption in the control WLM. As regards the supplemented WLMs, the correlation is not very clear as a consequence of the protease activity of *O. oeni*. Overall, the WLM with the two *S. cerevisiae* strains and *M. pulcherrima* Flavia presented higher amino acid incorporation since their extracellular concentration decreased. In contrast, WLM-TdViniferm generally showed an increase in all the analysed compounds.

Optimisation indexes for MLF performance

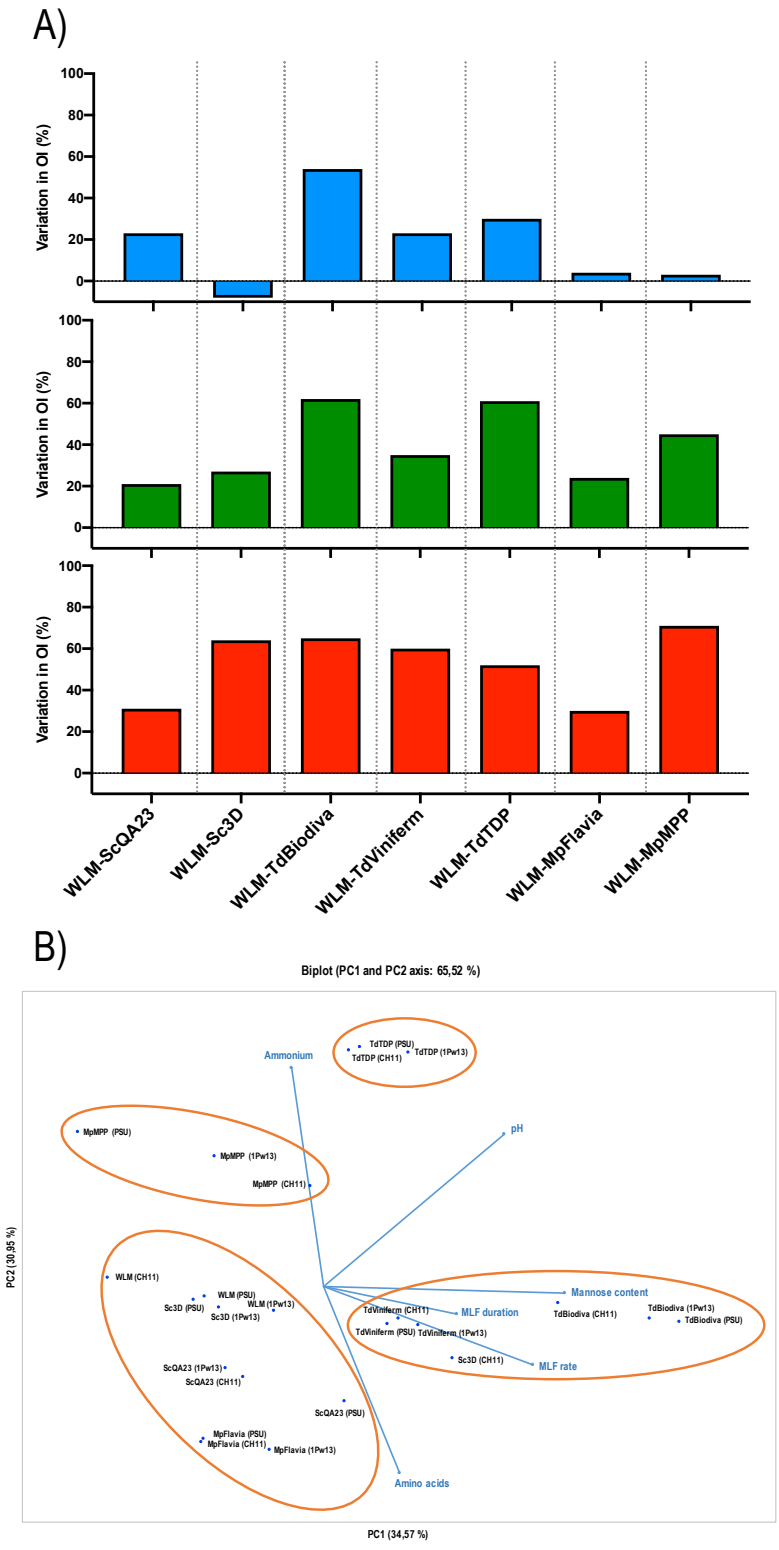


Figure 23. Optimization indexes. A) Percentage variation in the optimization index (OI) with respect to the control WLM for the three *O. oeni* strains: PSU-1 (blue), 1Pw13 (green) and CH11 (red). B) Principal component analysis (PCA) biplots of varimax rotated PCA of OI for each *O. oeni* strain in the different wines on which observations and variables are plotted.

To better determine the suitability of the yeast lees for each *O. oeni* strain, the optimization indexes (OI) were calculated. This allowed us to integrate the yeast lees' contribution to the chemical composition of the synthetic wines with the output of *O. oeni* in the MLF performance (Figure 23). In general, the OI increased in WLMs supplemented with yeast lees with respect to the control WLM. This behaviour was noticeable in *O. oeni* 1Pw13 and CH11 (Figure 23A). Meanwhile the variation as regards the *O. oeni* PSU-1 OI presented higher heterogeneity depending on the yeast lees. The OI only decreased in WLM-Sc3D for this *O. oeni* strain. Also, little variation was observed in the *M. pulcherrima* supplemented WLMs. The greatest increases in OI for all the *O. oeni* strains were obtained with the *T. delbrueckii* yeast lees, remarkably for Biodiva strain (Figure 23A). According to the PCA of the OIs (Figure 23B), the parameters of MLF performance (MLF duration and MLF rate) are related to higher concentrations of mannoproteins (mannose eq.), where the OIs of *T. delbrueckii* Biodiva and Viniferm for all the *O. oeni* strains are plotted. The opposite side of the PCA (Figure 23B) is where the OIs with the lowest values are plotted, relating to *S. cerevisiae, M. pulcherrima* and the control WLM.

Conclusions

The present study on MLF performance in the presence of simulated yeast lees in synthetic medium has shown that MLF may be positively or negatively affected by the presence of yeast lees and that this is strongly dependent on the *O. oeni* strain used. The highly heterogenous behaviour observed shows complex compatibility patterns between the yeast lees and *O. oeni*, as it occurs with wines fermented by different yeasts. The duration of MLF can be modified in the presence of yeast lees. In this study, the best MLF performance was observed in fermentations supplemented with one *T. delbrueckii* strain. This could be related to more favourable conditions for MLF associated to the addition of this yeast lees, such as a higher pH and a higher mannoprotein concentrations. In this regard, mannoprotein concentrations were increased with the addition of *T. delbrueckii* lees with respect to *S. cerevisiae* lees. In some cases, the consumption of mannoproteins could be related to a better malolactic performance. The protein and amino acid metabolism of each *O. oeni* strain comes about in response to the particular characteristics of the wine. In conclusion, further

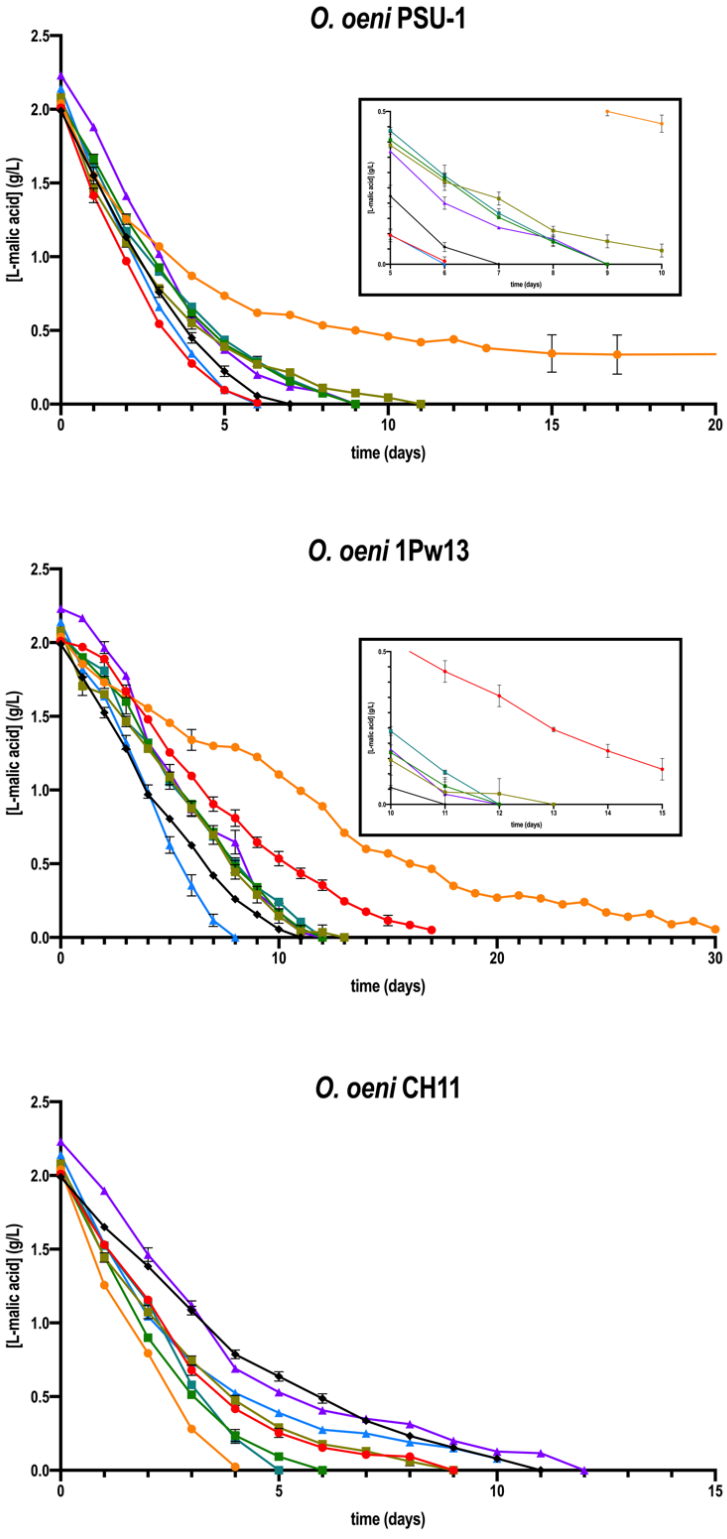
research is needed to understand yeast-bacteria strain compatibility, which is key to propose oenological practices to improve MLF performance.

Acknowledgments

We would like to thank Paloma Toraño for the adjustments made to mannoprotein precipitation and the mannose release protocol.

Supplementary Figures

Suppl. Figure S5. MLF performance of the three *O. oeni* strains as consumption of L-malic acid during their time in the different wine-like media (WLM). (-◆-) WLM, (-●-) WLM-ScQA23, (-●-) WLM-Sc3D, (-■-) WLM-TdBiodiva, (-■-) WLM-TdViniferm, (-■-) WLM-TdTDP, (-▲-) WLM-MpFlavia, (-▲-) WLM-MpMPP. MLF was considered to be finished when [L-malic acid] < 0.1 g/L.



CHAPTER III: 2

Use of yeast mannoproteins by *Oenococcus oeni* during malolactic fermentation under different oenological conditions

Aitor Balmaseda[a], Laura Aniballi[a], Nicolas Rozès[b], Albert Bordons[a*], Cristina Reguant[a]

* Corresponding author: albert.bordons@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

Submitted to Foods

Abstract

Oenococcus oeni is the main agent of malolactic fermentation in wine. This fermentation takes place after alcoholic fermentation, in a low nutrient medium where ethanol and other inhibitor compounds are present. In addition, some yeast-derived compounds such as mannoproteins can be stimulatory for *O. oeni*. The mannoprotein concentration in wine depends on the fermenting yeasts, and non-*Saccharomyces* in particular can increase it. As a result of the hydrolytic activity of *O. oeni*, these macromolecules can be degraded and the released mannose can be uptaken and used as an energy source by the bacterium. Here we look at mannoprotein consumption and the expression of four *O. oeni* genes related to mannose uptake (*manA, manB, ptsI* and *ptsH*) in a wine-like medium supplemented with mannoproteins and in natural wines fermented with different yeasts. We observe a general gene upregulation in response to wine-like conditions and different consumption patterns in the studied media. *O. oeni* was able to consume mannoproteins in all the wines. This consumption was notably higher in natural wines, especially in *T. delbrueckii* and *S. cerevisiae* 3D wines, which presented the highest mannoprotein levels. Regardless of the general upregulation, it seems that mannoprotein degradation is more closely related to the fermenting medium.

Keywords

Oenococcus oeni; non-*Saccharomyces*; mannoproteins; malolactic fermentation; gene expression

Introduction

Malolactic fermentation (MLF) is a biotransformation undergone in fermented beverages with low nutrient composition by different lactic acid bacteria (LAB) (Lerm et al., 2010). It is usually performed in wine and cider and results in an increase in pH values as a consequence of the decarboxylation of L-malic acid into L-lactic acid (Davis et al., 1985). As it occurs in fermented media, the fermenting microbiota – mainly yeasts that undergo alcoholic fermentation (AF) – will have a significant impact on the development of the MLF (Beltran et al., 2002). As a result of the transformation of grape must into wine (or apple juice into cider), the media in which the LAB will ferment will have low concentrations of nutrients, high acidity and high concentrations of ethanol and sulfur dioxide. All these changes will select the LAB most suited to this stressful environment, of which *Oenococcus oeni* is the best adapted species (Lonvaud-Funel, 1999). Since MLF usually takes places after AF, the interactions between yeasts and *O. oeni* will have a great impact on the development of the secondary fermentation (Balmaseda et al., 2018).

These two fermentative processes are usually inoculated with the species best adapted to each fermentation, *Saccharomyces cerevisiae* and *O. oeni* for AF and MLF respectively (Ribéreau-Gayon et al., 2006). Today there is increasing interest in the use of certain non-*Saccharomyces* yeasts – a group of yeasts naturally occurring in the first AF stages – as culture starters (Padilla et al., 2016b). Non-*Saccharomyces* are inoculated together with a selected *S. cerevisiae* strain to ensure completion of AF.

This vast group of yeasts known as non-*Saccharomyces* includes various species such as *Torulaspora delbrueckii, Metschnikowia pulcherrima, Starmerella bacillaris* and others (Petruzzi et al., 2017). Taking those wines fermented with *S. cerevisiae* as a reference, these species are associated with different chemical modulations such as lowering ethanol concentration (Belda et al., 2015; **Chapter I: 2;** Contreras et al., 2014), reducing medium chain fatty acids (MCFA) and sulfur dioxide concentration (Benito, 2018a; **Chapter I:2**) and increasing wine volatile compounds and mannoprotein concentration (Belda et al., 2015, 2016; Benito, 2018a; Romano et al., 2003) as well as other effects. (Fleet, 2008; Padilla et al., 2016b). These modulations are usually related to better MLF performance, as there is a decrease in inhibitory compounds and an

increase in stimulatory compounds for *O. oeni* (Balmaseda et al., 2018; Martín-García et al., 2020).

Among other things, the increase in mannoprotein concentration, especially through the use of *T. delbrueckii,* is stimulatory for *O. oeni* in wine MLF (Balmaseda et al., 2018; Guilloux-Benatier et al., 1995, 1998). Mannoproteins are found in yeast cell walls and are the main polysaccharide released during AF and ageing over lees (Giovani et al., 2012). They are formed by up to 80-90 % of monosaccharides, mainly mannose and traces of glucose, and around 10-20 % of amino acid residues (Giovani et al., 2012; Vejarano, 2020).

During the winemaking process they can be released from the yeast cell wall, especially during ageing, as a result of the yeast autolytic process (Belda et al., 2016; Vejarano, 2020). Mannoproteins seem to play an important role in MLF development by adsorbing the MCFAs produced by the yeasts and phenolic compounds of grape must and stimulating bacterial growth (Diez et al., 2010; Guilloux-Benatier et al., 1995; Liu et al., 2017b). It has also been demonstrated that *O. oeni* has glycosidase and peptidase activities that enable the release of sugars and amino acids from mannoproteins and other macromolecules, thereby increasing the nutritional content and the survival of *O. oeni* in wine (Alexandre et al., 2004). Mannose, which is released from yeast mannoproteins as a result of mannosidase activity by *O. oeni*, may be a phosphotransferase system (PTS) substrate, which may then be involved in the stimulation of LAB growth in the presence of yeast mannoproteins or yeast extracts (Jamal et al., 2013).

The main function of PTS is to translocate sugars across a membrane with simultaneous phosphorylation but without involving the concentration gradient (Jamal et al., 2013). PTS consists of several components including the enzyme I (EI), the histidine phosphocarrier protein (HPr) used in the phosphorylation cascade, and the substrate-specific permeases (enzyme II). The *ptsI* and *ptsH* genes encoding the general PTS proteins EI and HPr are highly conserved in the species. The enzyme II (EII) complex, which forms a mannose-specific permease, consists of two hydrophilic domains (domains A and B) and one or two hydrophobic integral membrane domains (domains C and D). The genes *manA* and *manB* are widely found in *O. oeni* strains,

whereas *manC* is more variable. EI and HPr (*ptsI* and *ptsH*) work with all sugars for the development of the phosphorylation cascade. EII is more specific of substrate but, apart from any preferred substrate such as mannose for *manA or manB*, it can also be active with other sugars such as glucose (Cibrario et al., 2016).

The possible use of the mannoproteins released from yeasts by *O. oeni* during MLF has not been thoroughly addressed. The aim of this paper is to evaluate the ability of *O. oeni* to utilize yeast mannoproteins in different fermentation media and to study the transcriptional response of mannose-related genes as possible indicators of mannose consumption.

Materials and methods

Microorganisms

The yeast strains used were *T. delbrueckii* Viniferm NS-TD (Agrovin, Alcázar de San Juan, Spain) (TdViniferm), *T. delbrueckii* Zymaflore Alpha (Laffort, France) (TdZymaflore), *M. pulcherrima* Flavia (Lallemand Inc., Montréal, Canada) (MpFlavia), *S. cerevisiae* Viniferm-3D (Agrovin, Spain) (Sc3D) and *S. cerevisiae* Lalvin-QA23 (Lallemand Inc.) (ScQA23). For MLF, *O. oeni* PSU-1 (ATCC BAA-331) was chosen because the primers used in the transcriptional study are based on this strain's genome sequence. Yeasts were maintained on YPD plates (2% glucose, 2% bacto-peptone, 1% yeast extract, 2% agar, w/v, Panreac Química SLU, Castellar del Vallès, Spain) and the bacteria on MRSmf (Margalef-Català et al., 2017b) plates, and all of them were stored at 4 °C.

Experimental fermentations

Fermentations in wine-like medium

Fermentations were carried out in a wine-like medium (WLM) and natural grape must. The fermentations in WLM were performed in 50 mL tubes containing 50 mL of sterile WLM, which was prepared as in Bordas et al. (2015) with half nitrogen composition (1.25 g/L of casamino acids and 1.25 g/L of peptone). This model wine had a concentration of around 110 mg N/L of yeast-assimilable nitrogen (YAN), 2 g/L of L-malic acid and a pH of 3.4. A stock solution (40 g/L) of the commercial mannoprotein extract Mannoplus (Agrovin, Spain) was prepared in WLM under

sterile conditions and the appropriate volume was added to each wine to obtain final concentrations of 0, 100, 200 and 400 mg/L of mannoprotein extract. Each wine (50 mL) was then inoculated with *O. oeni* PSU-1 for a population of around 2 x 10^7 cells/mL. These fermentations were carried out in triplicate and incubated statically at 20 ºC. Samples were taken every 24h to monitor the evolution of L-malic acid consumption and the bacterial population. This was calculated by plating samples on MRSmf and incubating at 27 ºC in a 10% CO_2 atmosphere for 7 days. MLF was considered to have finished when L-malic acid was below 0.1 g/L.

Fermentations in natural grape must

These were performed with natural concentrated Airén must (Mostos S.A., Tomelloso, Spain) diluted with sterile MiliQ water to a density of 1080 ± 1 g/L (Martín-García et al., 2020). The must was supplemented with 0.4 g/L of Nutrient Vit NatureTM (Lallemand Inc.) and pH was adjusted to 3.6. The must was then sterilized using 0.1% (v/v) of dimethyl dicarbonate (Santa Cruz Biotechnology, Inc., Dallas TX, USA) and stored overnight at 4 ºC.

In order to undergo the fermentations, precultured yeasts in YPD liquid medium were inoculated for a population of 2 x 10^6 cell/mL. In the case of non-*Saccharomyces*, after 48h from the initial inoculation, ScQA23 was inoculated for the same population. Fermentations were carried out statically in 500 mL of must, at 20 ºC and in triplicate. Every 48h, density (Densito 30PX Portable Density Meter, Mettler Toledo, Spain) and yeast population were determined. YPD agar plates were used to calculate the total number of yeast cells present, and lysine agar medium (Oxoid LTD., Basingstoke, UK) was used to quantify the non-*Saccharomyces* yeasts, after incubation at 28 ºC for 48 h. AF was considered to have finished when the sugar concentration was below 2 g/L. At this point the wines were centrifuged at 10,000 g for 5 min at 4 °C, filtered (0.22 µm Whatman, Thermo Fisher Scientific, Waltham MA, USA) and transferred to sterile 50 mL tubes. The wines were then inoculated with *O. oeni* PSU-1 for a population of 2 x 10^7 CFU/mL and incubated in the same conditions as the AFs. These fermentations were also carried out in triplicate. Samples were taken every 24 h to monitor the consumption of L-malic acid and the evolution of the bacterial population, as described above.

Wine characterization

Wines were characterized after AF and MLF. pH was measured with a pH-meter MicropH 2002 (Crison-Hach Lange, L'Hospitalet, Spain) and various compounds (primary amino nitrogen (NOPA), NH_4, acetic acid, citric acid, L-lactic acid, L-malic acid, D-lactic acid and glucose + fructose) were analyzed with a multianalyzer Miura One (TDI SL, Gavà, Spain). The mannoprotein content of WLM before and after MLF was quantified using a D-mannose and D-glucose assay kit K-MANGL (Megazyme, Wicklow, Ireland) as described in **Chapter III: 1**.

Sampling and RNA extraction

Cell pellets of *O. oeni* were collected during the fermentations. In the case of WLM, samples were collected at 1, 5 and 8 days after inoculation, corresponding to 24h, end of MLF and three days after the end of MLF respectively. Sampling of the natural grape fermentations was performed at the end of MLF. Cells of *O. oeni* grown in MRSmf for 2 days were also collected. In all cases, 50 mL of wine were centrifuged (4,250 g, 15 min) at 4 ºC. The supernatant was discarded and the cell pellet was frozen with liquid nitrogen and stored at -80 ºC until analysis. The cell pellet was then washed twice with 1 mL of sterile 10 mM Tris-HCl buffer at pH 8 by centrifugating at 2,000 x *g* for 5 min at room temperature. Afterwards, the cell pellet was resuspended in 200 µL of the same Tris-HCl buffer with 50 mg/mL of lysozyme (Roche Life Science, Mannheim, Germany) and incubated for 30 min at 37 °C. Finally, the RNA was purified using a High Pure RNA Isolation Kit Version 13 (Roche Life Science) following the manufacturer's instructions. The RNA was stored at -80 ºC until analysis.

RT-qPCR

The RNA samples were cleaned of contaminant DNA traces with a TURBO DNA-free Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Reverse transcription (RT) and real-time qPCR were performed following Olguín et al. (2009) using QuantStudio 5 Real-Time PCR Systems (Thermo Fisher). Four genes (*manA, manB, ptsI and ptsH*) related to mannose intake by *O. oeni* were evaluated (Jamal et al., 2013). In addition, another four genes (*dnaG, dpoIII, gyrA* and *gyrB*) were evaluated as internal controls. Of these, *gyrA* and *gyrB* presented the least variation within conditions (data not shown) and were therefore used as reference genes in this

experimentation. The primers used for all the genes studied can be found in Suppl. Table S6. The ΔΔCt method (Livak and Schmittgen, 2001) was used to calculate the relative expression of each gene. The expression of *O. oeni* after growing for 2 days in MRSmf was used as the reference condition in WLM. To study the differences in this metabolism through the use of non-*Saccharomyces* in natural wines, the expression of *O. oeni* in ScQA23 wine was used as a control condition.

Statistical analysis

All the statistical analyses of the results were performed using the statistics software XLSTAT version 2020.2.3 (Addinsoft, Paris, France). The analysis of variance was carried out by ANOVA with a subsequent Tukey HSD test to determine significant differences between the samples. The confidence interval used was 95% and the statistical level of significance was set at $p \leq 0.05$.

Results and discussion

Fermentations

All the MLF performed with *O. oeni* PSU-1 in WLM and natural grape must were completed. Information about the development of AF in natural grape must can be found in Suppl. Figure S7.

The addition of mannoproteins in WLM did not change the duration or consumption rate of L-malic acid (Table 16). In all the conditions, MLF took 5 days to finish. As for the consumption rate, only the addition of 100 mg/L of mannoprotein extract showed a significant increase in this value (Table 16). Under the studied conditions, the hypothesized positive effect of mannoproteins on MLF (**Chapter III: 1**) was not observed.

More differences were observed in natural grape wine MLFs. In these media, *O. oeni* showed a reduction in MLF length in *T. delbrueckii* and Sc3D wines with respect to the control ScQA23 wine (Table 17). The L-malate consumption rate increased by more than 25% in these wines. The MLF of *M. pulcherrima* wine was similar to the control condition. In general, the use of non-*Saccharomyces* promoted the MLF of *O. oeni* (Balmaseda et al., 2018). However, the fermentation with MpFlavia did not show this effect.

Table 16. Malolactic fermentation (MLF) parameters and concentration of various oenological compounds in wine like medium. MLF and post-MLF refer to the sampling point after MLF and 3 days of the completion of MLF. respectively.

		No addition	100 mg/L	200 mg/L	400 mg/L
Consumption rate (g/L·day)*		0.48 ± 0.02^{b}	0.55 ± 0.02^{a}	0.49 ± 0.02^{b}	0.48 ± 0.02^{b}
Duration (days)		5	5	5	5
pH	MLF	3.60 ± 0.01^{a}	3.60 ± 0.02^{a}	3.59 ± 0.01^{a}	3.61 ± 0.01^{a}
	Post-MLF	3.61 ± 0.0^{a}	3.61 ± 0.01^{a}	3.61 ± 0.01^{a}	3.61 ± 0.01^{a}
Glucose+Fructose (g/L)	MLF	1.63 ± 0.01^{a}	1.63 ± 0.03^{a}	1.65 ± 0.06^{a}	1.65 ± 0.02^{a}
	Post-MLF	1.63 ± 0.02^{a}	1.63 ± 0.03^{a}	1.68 ± 0.02^{a}	1.68 ± 0.04^{a}
Citric acid (g/L)	MLF	0.43 ± 0.03^{a}	0.41 ± 0.03^{a}	0.43 ± 0.03^{a}	0.45 ± 0.03^{a}
	Post-MLF	0.27 ± 0.08^{a}	0.31 ± 0.02^{ab}	0.35 ± 0.04^{ab}	0.37 ± 0.04^{b}
Acetic acid (g/L)	MLF	0.31 ± 0.01^{a}	0.33 ± 0.01^{a}	0.3 ± 0.01^{a}	0.29 ± 0.01^{a}
	Post-MLF	0.31 ± 0.01^{a}	0.32 ± 0.01^{a}	0.33 ± 0.01^{a}	0.32 ± 0.02^{a}
D-lactic acid (mg/L)	MLF	20.2 ± 1.2^{b}	40.5 ± 7.3^{a}	37.9 ± 9^{a}	38.5 ± 3.2^{a}
	Post-MLF	22.8 ± 0.6^{b}	49.5 ± 1.3^{a}	51.8 ± 1^{a}	56.1 ± 4.5^{a}

a–b Values are significantly different at $p \leq 0.05$. according to a Tukey post-hoc comparison test. * Calculation based on consumption rate of L-malic acid (MLF) considering the period of exponential decrease of this compound.

Mannoprotein utilization

We studied mannoprotein utilization by *O. oeni* during MLF in wine by precipitating the total polysaccharide fraction and quantifying it as the concentration in mannose equivalents after acidic hydrolysis before and after MLF. This procedure allowed us to estimate the concentration of mannoproteins (Ferrando et al., 2020) that were degraded during MLF (**Chapter III: 1**).

As regards WLM, a very low concentration of mannose equivalents (mannose eq.) was detected (Figure 24). This concentration was significantly increased by the addition of the commercial mannoprotein extract. We observed that the addition of this extract brought about a linear increase in the mannose concentration we quantified (data not shown). This corresponded to a mannose eq.: mannoprotein extract ratio (in mg/L) of around 0.23.

In all cases the mannoprotein concentration detected decreased by the end of MLF (Figure 24). We also quantified it 3 days after completion of MLF to better understand the metabolism in this synthetic model medium. In this post-MLF sampling we

observed a dramatic reduction compared to the previous samplings (Figure 24). It seems that when L-malic acid is completely metabolized, the utilization of mannoproteins increases. As a result, we can relate the utilization of mannoproteins to a survival metabolism that is enhanced when the preferred substrate of *O. oeni* in wine, L-malic acid, is drained. It is also notable that this decrease was related to the initial mannoprotein concentration. The higher the initial mannoprotein concentration, the higher the degradation in this post-MLF sampling (Figure 24).

Table 17. Malolactic fermentation (MLF) parameters and concentration of various oenological compounds in natural grape wines. AF and MLF refer to the sampling point after alcoholic fermentation and MLF. respectively. YAN means yeast assimilable nitrogen.

		ScQA23	Sc3D	TdViniferm	TdZymaflore	MpFlavia
Consumption rate (g/L·day)*		0.53 ± 0.02^{b}	0.80 ± 0.02^{a}	0.73 ± 0.02^{a}	0.76 ± 0.02^{a}	0.50 ± 0.02^{b}
Duration (days)		4	2	2	2	4
YAN (AF)	NOPA	34.93 ± 6.9^{ab}	24.36 ± 0.76^{b}	23.71 ± 2.68^{b}	28.59 ± 5.09^{ab}	37.29 ± 2.85^{a}
	NH_4	17.67 ± 1.53^{a}	13.67 ± 2.08^{a}	16.00 ± 2^{a}	13.67 ± 1.15^{a}	13.33 ± 1.15^{a}
pH	AF	3.49 ± 0.04^{ab}	3.47 ± 0.01^{ab}	3.42 ± 0.01^{a}	3.44 ± 0.02^{a}	3.54 ± 0.04^{b}
	MLF	3.77 ± 0.04^{d}	3.67 ± 0.01^{bc}	3.59 ± 0.01^{a}	3.62 ± 0.01^{ab}	3.71 ± 0.01^{c}
Glucose+Fructose (g/L)	AF	n.d.^{a}	1.66 ± 0.48^{b}	1.05 ± 0.26^{ab}	1.55 ± 0.14^{ab}	1.41 ± 0.01^{ab}
	MLF	n.d.^{b}	1.67 ± 0.07^{a}	0.47 ± 0.38^{a}	0.61 ± 0.21^{a}	1.12 ± 0.57^{a}
Citric acid (g/L)	AF	0.59 ± 0.02^{ab}	0.62 ± 0.03^{b}	0.69 ± 0^{c}	0.73 ± 0.02^{c}	0.56 ± 0.02^{a}
	MLF	0.21 ± 0.02^{b}	0.11 ± 0.01^{a}	0.11 ± 0.02^{a}	0.11 ± 0.02^{a}	0.11 ± 0.01^{a}
Acetic acid (g/L)	AF	0.29 ± 0.02^{b}	0.28 ± 0.04^{b}	0.25 ± 0.04^{ab}	0.17 ± 0.04^{a}	0.59 ± 0.04^{c}
	MLF	0.70 ± 0.01^{b}	0.66 ± 0^{b}	0.47 ± 0.04^{a}	0.43 ± 0.07^{a}	0.74 ± 0.01^{b}
D-lactic acid (g/L)¶	MLF	0.20 ± 0.01^{b}	0.26 ± 0.06^{cd}	0.29 ± 0.01^{d}	0.24 ± 0^{c}	0.14 ± 0.01^{a}
Ethanol (% vol/vol)	AF	10.8 ± 0.2^{a}	11 ± 0.2^{a}	11.2 ± 0.2^{a}	10.7 ± 0.2^{a}	10.8 ± 0.2^{a}

a–d Values are significantly different at $p \leq 0.05$ according to a Tukey post-hoc comparison test. * Calculation based on consumption rate of L-malic acid (MLF) considering the period of exponential decrease of this compound. n.d.: not detected. ¶ D-lactic acid was not detected in wines after AF.

The results for mannoprotein utilization during MLF in natural grape wine can be found in Figure 25. Considering ScQA23 wine as the control condition, we observed a significant increase in mannoprotein concentrations in *T. delbrueckii* wines and when inoculating Sc3D. Similar values of around 340 mg /L of mannose eq. were quantified in TdViniferm and Sc3D wines. As regards TdZymaflore wine, the mannoprotein concentration detected was the highest (440 mg/L eq. of mannose on average). Thus *T. delbrueckii* is a non-*Saccharomyces* yeast related to an increase in mannoprotein

concentrations (Belda et al., 2016; Benito, 2018a; Ferrando et al., 2020) in its fermented wines and also when added as yeast lees in a synthetic medium (**Chapter III: 1**). In addition, Sc3D is a selected commercial strain that is an overproducer of these macromolecules (Belda et al., 2016).

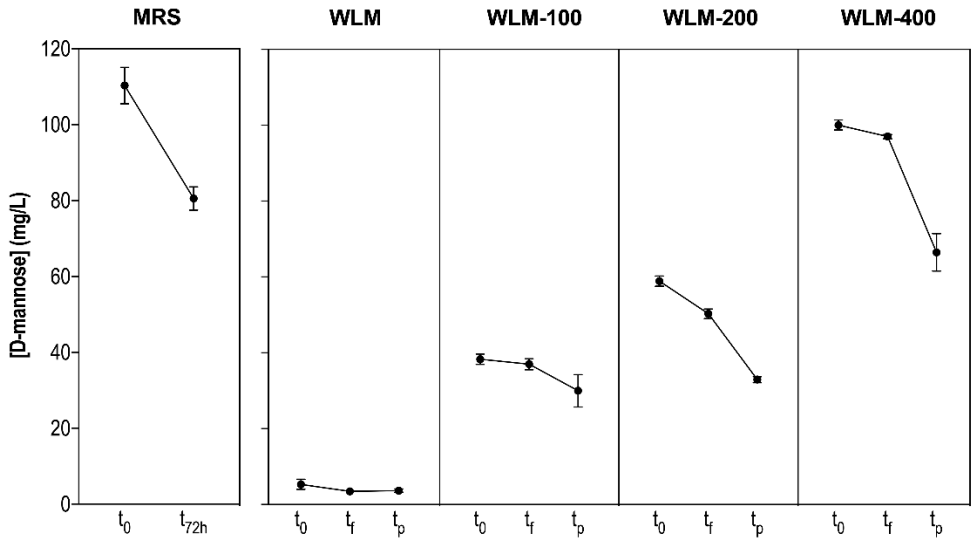


Figure 24. Mannoprotein concentration (mg of mannose eq./L) in wine-like medium (WLM) with mannoprotein extract addition throughout malolactic fermentation of *O. oeni* PSU-1. WLM-100, -200 and -400 represent the concentration (mg/L) of commercial mannoprotein extract added. t_0, t_f and t_p represent before *O. oeni* inoculation, at the end of MLF ([L-malic acid] < 0.1 g/L) and after MLF (3 days after completion of MLF) respectively.

In *T. delbrueckii* and Sc3D wines, in which the concentration of mannoproteins after AF and mannose utilization by *O. oeni* were the highest (Figure 25), the L-malic acid consumption rate was also the highest (Table 17). We can therefore relate better MLF performance to enhanced mannoprotein utilization. It is interesting to note that the same *O. oeni* strains exhibit different use patterns depending on the yeast inoculated and the amount of mannoprotein released during AF.

In the case of MpFlavia, however, no differences were observed with respect to the control (280 mg/L of mannose eq. on average) even though this yeast – and this strain in particular – usually increases the mannoprotein concentration in wines (Belda et al., 2016; Ferrando et al., 2020) or produces similar concentrations to *S. cerevisiae*. The interactions of the yeast with the medium in which it is fermenting presumably determine the release of mannoproteins into the medium, probably related to the particular autolytic process in that medium (Loira et al., 2014). In addition, *M.*

pulcherrima and *S. cerevisiae* wines showed the lowest L-malic acid consumption rate (Table 17), which is also related to less mannoprotein release and utilization by *O. oeni*, supporting the idea that better MLF performances may be related to greater mannoprotein degradation (Figure 25).

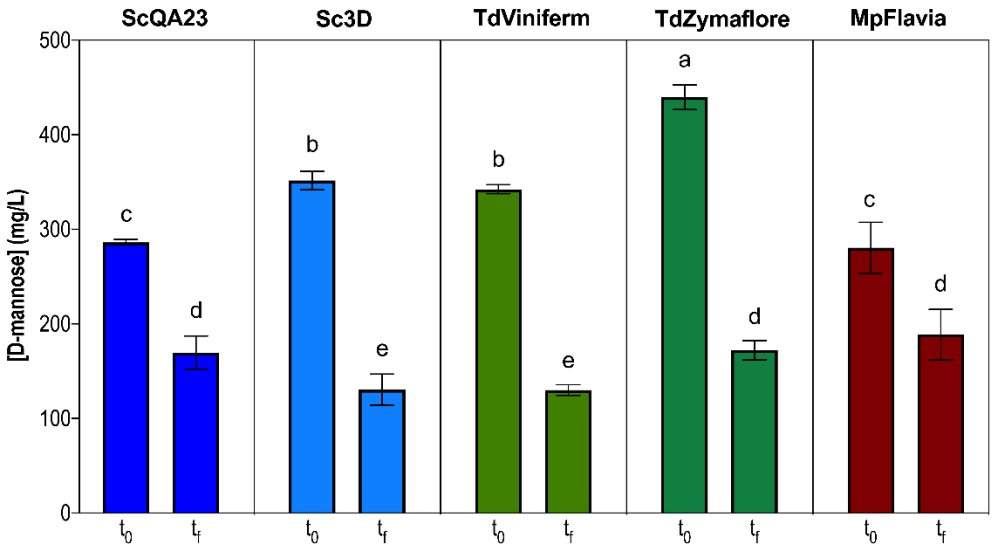


Figure 25. Mannoprotein concentration (mg of mannose eq./L) before and after malolactic fermentation in natural grape wines produced following different yeast inoculation strategies. Sc, Td and Mp represent *S. cerevisiae, T. delbrueckii* and *M. pulcherrima* respectively, followed by the name of the commercial strain. t_0 and t_f represent before *O. oeni* inoculation and at the end of MLF ([L-malic acid] < 0.1 g/L).

The use of mannoproteins was different in the two types of wine studied. In natural grape wines the highest mannoprotein degradation was detected once the L-malic acid was exhausted. This is probably because mannoprotein concentration is higher in natural wines and also because other inhibitor compounds such as yeast metabolites or less nutrients may be present in these wines. In fact, the YAN (Table 27) in natural wines was less than the half the WLM (around 110 mg N/L). Under these more limiting conditions the use of mannoproteins is enhanced in *O. oeni*. Thus, the breakdown of mannoproteins would release amino acids, apart from mannose, which could eventually be used as a nitrogen source by *O. oeni*.

General wine chemical compounds

The MLF undergone with *O. oeni* in WLM transformed L-malic acid into L-lactic acid analogously in all wines (data not shown). We analyzed the chemical composition after MLF and at the post-MLF sampling point (Table 16). No differences were observed within the conditions and sampling points as regards pH. Sugar

concentration decreased slightly as a consequence of MLF. Nevertheless, the behavior in all WLM was similar and no sugar decrease was observed in the post-MLF sampling. Similarly, citric acid was not consumed by *O. oeni* in these conditions, neither after MLF nor post-MLF. Citric acid decreased slightly at the post-MLF sampling point as a consequence of the consumption of the main carbon source, L-malic acid, being drained. The consumption of citric acid was around 0.1 g/L in all cases (Table 16) and no significant changes were detected in acetic acid concentration as this is usually related to sugar or citric acid consumption. D-lactic acid, which can be an end product of sugars in forms such as glucose, fructose or mannose – which we can also relate to mannoprotein metabolism – was detected in higher concentrations in those wines to which the mannoprotein extract was added. Even when the concentrations were low after MLF, the addition of the mannoprotein extract resulted in a doubling of the D-lactic acid detected (Table 16). Moreover, in the post-MLF sampling the concentrations increased in all wines, whereas no significant increase was observed in the WLM without the addition.

More differences were observed in the compounds studied in the natural wines (Table 17). First, all the wines finished AF with similar L-malic acid concentrations of around 1.5 g/L (data not shown). The *T. delbrueckii* wines produced the most acidic wines, which were significantly different from the *M. pulcherrima* wine. The pH differences were as high as 0.1. As a result of MLF the pH value increased, and the observed differences were also similar since they had similar amounts of L-malic acid. The residual sugar concentration (glucose + fructose) after AF was always below 2 g/L. Nevertheless, the wine inoculated with ScQA23 completely drained the sugars, whereas in the others, fermented with the other *S. cerevisiae* strain and sequential inoculations with non-*Saccharomyces,* sugar traces were detected. These residual sugars were slightly consumed by *O. oeni* by the end of MLF in those wines inoculated with non-*Saccharomyces*, particularly those fermented with *T. delbrueckii*. In these wines the citric acid concentration after AF was slightly different as a result of the yeasts' metabolism.

As mentioned earlier, the YAN concentration in the natural wines (Table 17) was lower than in WLM (110 mg N/L). The concentration of NOPA was significantly altered as a result of AF inoculation in the natural wines (Table 17). The *T. delbrueckii*

and Sc3D wines had lower NOPA concentrations than the ScQA23 and *M. pulcherrima* wines. This can be explained by the different amino acid consumption patterns and preferences (Roca-Mesa et al., 2020), which are also the result of yeast-yeast interactions (Bordet et al., 2020). However, the differences were small and all the wines had enough NOPA concentration to ensure MLF. In contrast, similar ammonium concentrations were also observed (Table 17).

The use of non-*Saccharomyces* is usually related to higher citric acid concentrations after AF (Balmaseda et al., 2018), although this has only been clearly observed with *Starmerella bacillaris* (Giaramida et al., 2013). In the present study, the wines fermented with *T. delbrueckii* showed a significant increase in citric acid concentrations compared to the *S. cerevisiae* control wine. After MLF the concentration of citric acid was reduced as a consequence of the consumption by *O. oeni*. All the wines had around 0.11 g/L of this acid after MLF, with the exception of the ScQA23 wine, which had twice the concentration (Table 17).

After AF, the acetic acid concentration was different in the obtained wines. As described in the literature, the use of some non-*Saccharomyces* can change the concentration of this compound (Martín-García et al., 2020; Padilla et al., 2016b). Generally speaking, *T. delbrueckii* tends to decrease it while *M. pulcherrima* increases it. In our study, *M. pulcherrima* significantly increased the volatile acidity of the wine after AF (up to 0.59 g/L on average) and only TdViniferm decreased it significantly (0.17 g/L on average). As a result of the consumption of sugars and citric acid by *O. oeni*, acetic acid and D-lactic acid are produced. After MLF, the concentration of acetic acid also depended on the combination of yeast species used in AF. The *S cerevisiae* fermented wines had the intermediate concentration of acetic acid, while the *T. delbrueckii* wines had the lowest and *M. pulcherrima* the highest.

D-lactic acid is a sugar related to the LAB metabolism and therefore it was not detected after AF. It increased as a result of the *O. oeni* metabolism after MLF (Table 17). The detected concentration was dependent on the mannoprotein concentration after AF (Figure 25), which resulted in higher D-lactic acid in the *T. delbrueckii* and Sc3D wines, 0.29 and 0.26 g/L on average respectively.

Transcriptional response of mannose-related genes

The transcriptional regulation of four selected genes (Suppl. Table S6) previously related to mannose uptake in *O. oeni* (Jamal et al., 2013) were evaluated in a synthetic medium with increasing concentrations of mannoprotein extract. This transcriptional regulation was compared to the expression of *O. oeni* prior to inoculation to determine the expression level of these genes under oenological conditions. The relative expression (RE) of the genes was quantified 24h after inoculation, at the end of MLF and 3 days after completion of the fermentation (Figure 26).

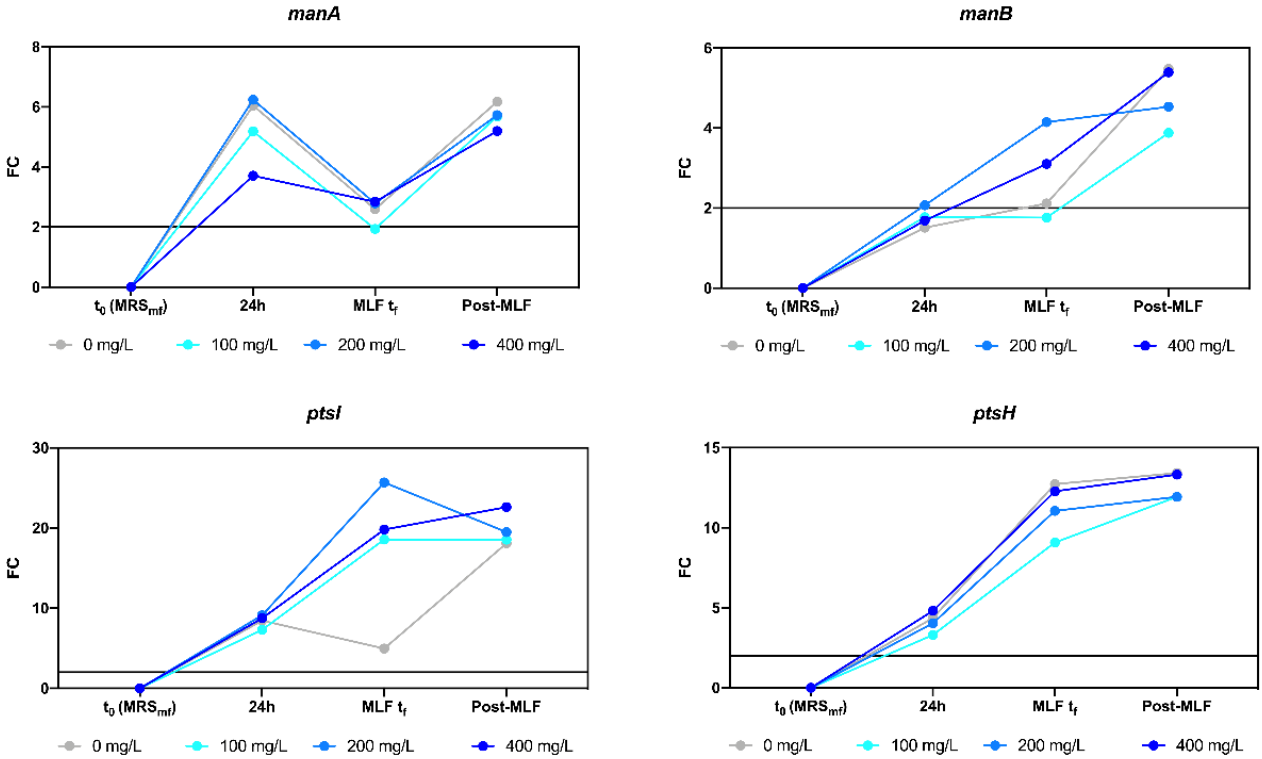


Figure 26. Evolution of relative expression, as fold change (FC), of *manA, manB, ptsI* and *ptsH* in WLM with different additions of mannoprotein extract, using the expression of the inoculum as the reference condition. FC=2 is shown in the graph as the threshold for considering a gene to be upregulated.

Studying the gene expression of mannose uptake-related genes under oenological conditions is difficult since their expression is also dependent on the concentrations of other sugars or sugar alcohols (Cibrario et al., 2016; Jamal et al., 2013; Kim et al., 2011). These genes are generally activated when growing in the presence of sugars (Cibrario et al., 2016) and under ethanol stress (Jamal et al., 2013). Their relationship with the pH is more variable. Jamal et al. (2013) observed that *manA* was expressed more in

acidic conditions, whereas *manB* was more active in neutral pH and *ptsI* and *ptsH* showed no variation.

In our study the genes *manA*, *ptsI* and *ptsH* were upregulated in response to WLM conditions 24h after inoculation. The expression of *manB* did not show any change compared to the control condition (before inoculation). The expression at the end of MLF was variable depending on the gene and mannoprotein concentration. However, there was a general increase after the end of MLF in all the studied conditions. Three days after L-malic acid exhaustion when most of the mannose consumption was detected, all the genes were upregulated compared to the control condition. It seems that when not many carbon sources are available, *O. oeni* increases the expression of these permeases to enable bacterial survival. Indeed, the bacterial population remained stable at around 10^7 CFU/mL 3 days after the completion of MLF (data not shown). This highlights the importance of using alternative energy sources after L-malic consumption in order to allow bacterial survival at least 3 days after completion of MLF.

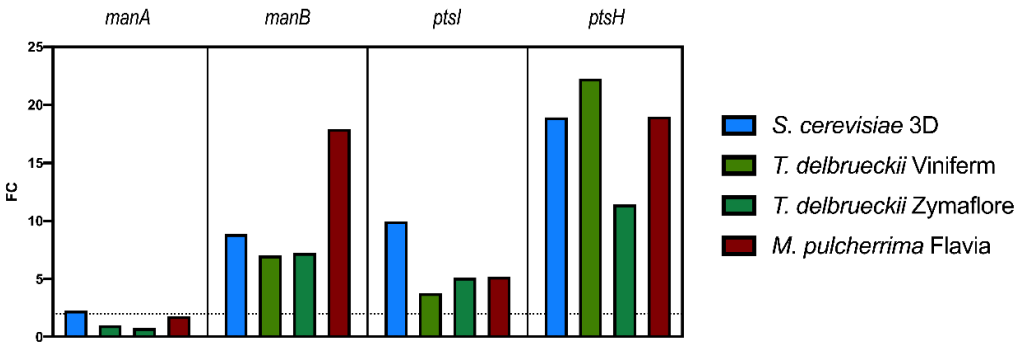


Figure 27. Relative expression, as fold change (FC), of *manA, manB, ptsI* and *ptsH* in natural wines fermented with different yeast inoculation regimes by the end of malolactic fermentation, using the expression in *S. cerevisiae* QA23 wine as the reference condition. FC=2 is shown in the graph as the threshold for considering a gene to be upregulated.

We also studied these four genes in wine from natural grape must with different AF inoculation regimes by the end of MLF (Figure 27). Taking the expression of these genes in ScQA23 wine as the reference conditions, *manB, ptsI* and *ptsH* were upregulated in the other wines. The gene *manA* did not show an expression pattern different from the reference condition with the exception of a slight upregulation in Sc3D wine.

It is interesting to see that the expression of these genes was upregulated in *M. pulcherrima* wine (Figure 27), which had low mannoprotein degradation levels (Figure 25) comparable to the reference condition. Therefore, the wine matrix must have an important effect on the expression of these genes, which makes it difficult to find any relation between RE and mannoprotein use. Moreover, it has to be remembered that these genes encode non-specific hexose permeases, which are usually active (Jamal et al., 2013) and respond not only to mannose. Thus, it should not be surprising that these genes may be upregulated in response to oenologically stressful conditions. Nevertheless, no correlation was found between the RE and mannoprotein use patterns in *O. oeni*. This suggests a complex regulation dependent on the medium and not specifically linked to the expression of mannose uptake-related genes.

Conclusions

This study presents new information on mannoprotein utilization by *O. oeni* during MLF under oenological conditions. Different mannoprotein concentrations were quantified following an AF inoculation strategy. *T. delbrueckii* wines together with Sc3D wines were those with the highest concentrations of mannoproteins released. Mannoprotein utilization by *O. oeni* was dependent on the fermenting media. Low degradation of mannoproteins was observed when fermenting in a low mannoprotein concentration medium (WLM), whereas this degradation was higher in natural wines, with a higher mannoprotein content. This greater utilization of mannoproteins may be associated with a faster MLF in *T. delbrueckii* and Sc3D wines. The genes *manA, manB, ptsI* and *ptsH* – directly related to mannose uptake but also active with other sugars – were upregulated in response to oenological conditions. *O. oeni* showed an increased RE of *manB, ptsI* and *ptsH* in non-*Saccharomyces* and Sc3D wine compared to ScQA23 wine. Altogether it seems that the mannoprotein metabolism is activated under oenological conditions and that mannoprotein uptake is enhanced in stressful conditions. Further research is needed to clarify the regulation of mannoprotein metabolism in *O. oeni*, seeking out other possible genes/proteins involved in this metabolism. The use of different yeasts and mannoprotein extracts should be further evaluated, with more *O. oeni* strains and conditions, as potential activators of MLF.

Acknowledgements

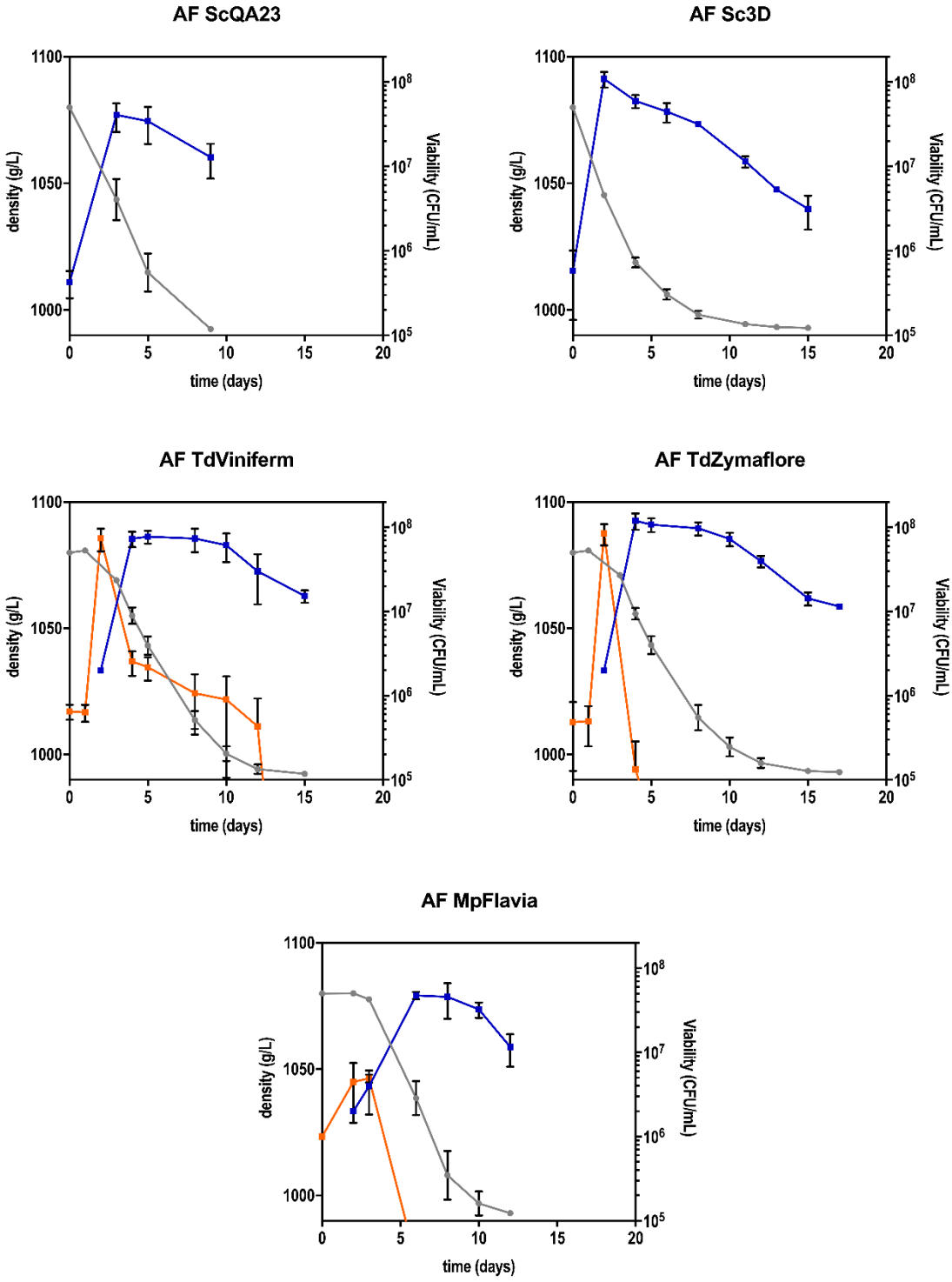
We would like to thank Paloma Toraño for the mannoprotein quantification protocol optimization. Aitor Balmaseda is grateful to the Catalan Government for the predoctoral fellowship (2018FI_B 00501). Laura Aniballi is grateful to the Erasmus+ program for the mobility grant from the University of Verona.

Supplementary Tables

Supplementary Table S6. Primers used in this work.

Gene	Sequence (5'→3')	Reference
manA	F- TTCATTGGCGCAGCCGGTTT	Jamal et al. 2013
	R- GCCGTTGCTAAAATCGTCCC	
manB	F- AGTCCAGTGGGCTTCTTTCT	Jamal et al. 2013
	R- TTGGTTCCAACGATTCAGGC	
ptsI	F- GACGAACAGCTCATGCTTCG	Jamal et al. 2013
	R- ATCGATTAAGACCTGGCCGG	
ptsH	F- CGATTACTGCTGACTCTGGC	Jamal et al. 2013
	R- TACCCGCGCCAAGACTCATT	
dpolIII	F- AATTCGCACGGATTGTTTTC	Stefanelli. 2014
	R- GCGAACCAGCATAGGTCAAT	
dnaG	F- TGTGGACGGAGTGGCAATGT	Desroche et al. 2005
	R- CAGTATTTTCTGTATATTTACTATCG	
gyrA	F- CGCCCGACAAACCGCATAAA	Desroche et al. 2005
	R- CAAGGACTCATAGATTGCCGAA	
gyrB	F- GAGGATGTCCGAGAAGGAATTA	Desroche et al. 2005
	R- ACCTGCTGGGCATCTGTATTG	

Supplementary Figures



Supl. Figure S7. Alcoholic fermentation dynamics where density decrease (grey) and yeast viability are represented for the used yeast species in each wine: *S. cerevisiae* (blue) and non-*Saccharomyces* (orange). Sc, Td and Mp represent *S. cerevisiae, T. delbrueckii* and *M. pulcherrima* respectively, followed by the name of the commercial strain. Values shown are the mean of triplicates ± SD.

CHAPTER IV

Oenococcus oeni molecular adaptation response in non-*Saccharomyces* fermented wines: comparative multiomic aproach

Aitor Balmaseda[a], Nicolas Rozès[b], Albert Bordons[a],

Cristina Reguant[a,*]

* Corresponding author: cristina.reguant@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

Manuscript in preparation

Abstract

Oenococcus oeni is the main agent responsible for wine malolactic fermentation (MLF). This fermentation is usually performed in high acidity and red wines after alcoholic fermentation (AF). The result of this AF produces a nutrient impoverished and harsh medium with high concentration of microbial inhibitor compounds as ethanol, medium chain fatty acids or SO_2. Under these conditions *O. oeni* has developed a highly specialised molecular mechanisms in order to be able to survive in wine. Current studies in non-*Saccharomyces* report a stimulatory effect on *O. oeni*, generally related with a decrease in the inhibitor compounds found in wine. In this work, we studied the molecular adaptation of *O. oeni* in wines fermented with *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, two of the most currently relevant non-*Saccharomyces* throughout a comparative multiomic approach. These results were compared to the adaptation of *O. oeni* in *S. cerevisiae* wine to determine the main changes due to the use of non-*Saccharomyces*. The duration of MLF was shortened by the use of non-*Saccharomyces*, to the half with *T. delbrueckii* and to the quarter with *M. pulcherrima*. In this work we observed for the first time how *O. oeni* responds at molecular level to the changes produced by non-*Saccharomyces*. We showed a differential adaptation of *O. oeni* in the studied wines. In this sense, the main molecular functions affected were amino acid and carbohydrate transport and metabolism, from which peptide metabolism appeared as a key metabolism under wine-like conditions. We also showed that the abundance of Hsp20, a well-known stress protein, was dependent of the duration time. Thus, the use of non-*Saccharomyces* reduced the abundance of Hsp20, that could be related to a less stressful wine-like condition for *O. oeni*.

Keywords

Non-*Saccharomyces*, malolactic fermentation, *Oenococcus oeni*, wine, proteomics, transcriptomics

Introduction

Oenococcus oeni is the main microbial agent responsible for wine malolactic fermentation (MLF) (Lonvaud-Funel, 1999). This process occurs naturally in wine, or it can be undergone by the inoculation of selected strains. MLF usually takes place after alcoholic fermentation (AF) driven out by oenological yeasts (Ribéreau-Gayon et al., 2006). During this process, yeasts transform grape must into wine. As consequence, there is consumption of sugars and other nutrients by yeasts that produce a very impoverished medium where *O. oeni* must propagate. Besides, during this process, other microbial inhibitor compounds are produced as high concentrations of ethanol, SO_2, and medium chain fatty acids, among others (Balmaseda et al., 2018; Bech-Terkilsen et al., 2020).

Traditionally, *Saccharomyces cerevisiae* has been used as starter culture for AF (Padilla et al., 2016b). Nevertheless, recent studies about non-conventional yeasts, those oenological yeasts different from *S. cerevisiae*, leaded to an increase in their use in winemaking. Indeed, different strains of non-*Saccharomyces* are currently commercially available, like strains of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* species (Capozzi et al., 2015; Roudil et al., 2020). Since the metabolism of the fermenting yeasts will determine the physiochemical conditions of wine after AF, they will directly affect the development of MLF (Balmaseda et al., 2018). In this sense, the use of some non-*Saccharomyces*, as the ones above, have been related with a mitigation of some inhibitory compounds, and thus, positively affecting MLF (**Chapter I: 1**; Martín-García et al., 2020).

Still, wine is a very harsh environment, which *O. oeni* must face to overcome those oenological stress factors (Bech-Terkilsen et al., 2020). The genetic particularities of this lactic acid bacterium (LAB) make it the best adapted bacterium in wine-like conditions (Beltramo et al., 2006). From those, stress response genes as *clp*, *grpE*, *groES*, *hsp18*, *hdc*, *ftsH*, *cfa*, and *trxA*, among others, appeared to be essential (Beltramo et al., 2004; Bourdineaud, 2006; Bourdineaud et al., 2003, 2004; Guzzo et al., 2000; Jobin et al., 1997; Margalef-Català et al., 2016a; Olguín et al., 2009, 2010; Spano and Massa, 2006). Moreover, other genes related with nitrogen metabolism and translation have been reported as essential to overcome wine conditions (Margalef-Català et al., 2016a).

The studies of these genes under wine conditions are important to better understand how they are activated or repressed. In this sense, the publication of the first complete genome of an *O. oeni* strain in 2005 (Mills et al., 2005) made possible the use of omic approaches for *O. oeni*. Since then, some authors have studied *O. oeni* adaptation mechanisms. Silveira et al. (2004) showed for the first time that the proteomic profile of *O. oeni* was different in ethanol shock or adaptation. Then, Cecconi et al. (2009) studied the proteomic profile in the adaptation to wine conditions. Also, Liu et al. (2017) studied the transcriptomic changes of acidic shock, and Sternes et al. (2017) studied the transcriptomic profiles of three *O. oeni* strains in wine. Besides, some works presented a combined transcriptomic and proteomic studies of *O. oeni*. Olguín et al. (2015) studied the ethanol shock after 1 h of incubation, Costantini et al. (2015) studied the adaptation to wine conditions at 24 h. Margalef-Català et al. (2016) studied the adaptation to wine conditions during the first 8 h in wine. And recently Yang et al. (2020) studied the combined effects of acidic and ethanol stresses.

In this context, and focusing on the use of non-*Saccharomyces*, we studied the adaptation transcriptomic and proteomic profile of *O. oeni* PSU-1 in wines fermented with *T. delbrueckii* and *M. pulcherrima*, in sequential inoculation with *S. cerevisiae*, and wine fermented only with *S. cerevisiae*. This new approach will show those molecular mechanisms affected by the use of these non-conventional yeasts in *O. oeni*.

Materials and methods

Microorganisms and inocula

The yeast strains used were *T. delbrueckii* Biodiva, *M. pulcherrima* Flavia and *S. cerevisiae* Lalvin-QA23, all from Lallemand Inc. (Montréal, Canada). Strain *O. oeni* PSU-1 (ATCC BAA-331) was used for the MLF. Yeasts were grown in YPD medium at 28 ºC (20 g/L glucose, 20 g/L bacto-peptone, 10 g/L yeast extract) and *O. oeni* in MRSmf (Margalef-Català et al., 2017b) at 27 ºC with a 10% atmosphere of CO_2. Yeasts and *O. oeni* were maintained on plates of the same media with 20 g/L agar and stored at 4 ºC.

Fermentation trials

The fermentation must was prepared using white grape concentrated (65.4 ° Brix; Mostos Españoles S.A., Tomelloso, Spain) and sterile Milli-Q purified water to obtain a density 1075 g/L (which corresponds to around 200 g/L of glucose and fructose). After the dilution with water, must was supplemented with nutrients (0.4 g/L Nutrient Vit Nature™, Lallemand Inc.) and the pH was adjusted to 3.6 with HCl 37 % (vol/vol). Finally, the must was sterilized by adding 1 % of dimethyl dicarbonate (Santa Cruz Biotechnology, Inc., Dallas TX, USA) and kept at 4ºC overnight.

Alcoholic fermentations were carried out in 5 L flasks containing 5 L of must statically at 20 ºC. Musts were inoculated with the two non-*Saccharomyces* strains: *T. delbrueckii* (Td) or *M. pulcherrima* (Mp), and inoculating *S. cerevisiae* after 48 h. Each yeast was inoculated for a population of 2.5 x 10^6 cells/mL previously grown in YDP liquid medium. There was also a control fermentation with *S. cerevisiae* as a sole starter (Sc). All fermentations were performed in triplicate. Samples were taken every 48 h to monitor density decrease and yeast population evolution. YPD agar medium was used to calculate the total number of yeasts, and lysine agar medium (Oxoid LTD., England) was used for quantification of non-*Saccharomyces* yeasts (Wang et al., 2015), after incubation at 28 °C for 48h. AF was considered finished when sugar (glucose + fructose) was below 2 g/L.

After AF wines were first centrifugated (8500 x *g* for 5 minutes) and then, filtered with a 0.22-micron pore size (Merck Millipore Steritop™ Sterile Vacuum Bottle-Top Filters, Madrid, Spain). Each AF replicate of 5 L was divided in two 2 L flasks of 2 L which will correspond to t_0 MLF and t_f MLF samples. Flasks were inoculated with *O. oeni* for a population of 2 x 10^7 cells/mL. These fermentations were also carried out in triplicate and, statically at 20 ºC. After 1h of incubation samples for omic analyses were taken from t_0 MLF flask.

Samples were taken every 24 h to monitor L-malic acid consumption and bacterial population. Samples were plated on MRSmf and incubated at 27 °C in a 10 % CO_2 atmosphere for 7 days. MLF was considered as finished when L-malic acid was below 0.1 g/L.

Sugar content at final stages of AF and L-malic acid during MLF were determined using the multianalyser Miura One (TDI SL, Gavà, Spain). On completion of AF and MLF, pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain) and primary amino nitrogen (NOPA), NH_4, citric acid, and acetic acid were quantified using the multianalyser Y15 (Biosystems, Barcelona, Spain). Ethanol concentration was also measured at the end of AF by ebulliometry (Electronic ebulliometer uEBU6576, GabSystem, Moja, Spain) in accordance with the Compendium of International Methods of Analysis of Musts and Wines (OIV, 2009).

Sampling for omics analyses

For the analyses of *O. oeni*, wines at the beginning of the MLF (1h after *O. oeni* inoculation, t_0 MLF flask) and at the end of MLF (when [L-malic acid] < 0.1 g/L, t_f MLF flask) were sampled. Appropriate volumes of wine were centrifugated at 4,600 x *g* for 20 minutes at 4 ºC for each analysis, 1 L for proteomic and 50 mL for transcriptomic analyses. The resulting pellet of 1 L of wine was washed with 10 mM Tris-HCl buffer at pH 8, frozen in liquid nitrogen and kept at −80 ºC until protein extraction. For transcriptomic analyses, the pellet of 50 mL of wine was washed with 10 mM Tris-HCl prepared with diethyl pyrocarbonate-treated water (DEPC), and then frozen in liquid nitrogen and kept at −80 ºC until RNA extraction.

RNA and protein extraction

RNA and protein extraction from *O. oeni* were performed following Margalef-Català et al. (2016). For RNA extraction, cell pellet was defrosted and washed again with 10 mM Tris-HCl DEPC water. High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for the extraction following manufacturer's instructions changing the cell lysis for lysozyme dissolved in 10 mM Tris-HCl buffer DEPC, at 50 mg/mL during 30 minutes at 37 ºC. Total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Bremen, Germany). Extracted RNA was stored at -80 ºC until RNA analysis.

Cell pellet for protein extraction were resuspended in 0.1M Tris-HCl at pH 7.5, mixed with protease inhibitor cocktail from Roche. Cells were disrupted using One-shot disruptor (Constant Systems Ltd.) at 5 ºC, applying twice a 2.7 kbar pressure.

Protein suspension was centrifuged at 4,500 x *g* for 15 minutes at 4 ºC to remove cell debris, protein concentration was estimated (Bradford, 1976) and the supernatant was frozen in liquid nitrogen until protein analysis.

Transcriptomic analyses

RNA of each sample was ribo-depleted using riboPOOL kit (TOOLs Biotech) according to manufacturer's protocol. Between 60 and 250 ng of ribo-depleted RNA were used to prepare strand-specific barcoded libraries with the Total RNA-seq v2 kit (catalog no. 4475936, Thermofisher). Each library was quantified by micro-fluid electrolysis in Agilent TapeStation using Agilent High Sensitivity Screen Tape kit. The barcoded libraries were amplified with Ion 540TM Kit –Chef (Ion Torrent) and pooled on 540 chips to be sequenced on GeneStudio S5 System (Ion Torrent). Low quality reads (phred < 17) were filtered prior to analysis as well as reads that present 6bp length homopolymers. Reads were mapped against reference genome *Oenococcus oeni* (NC_008528.1) using HISAT2 (V.2.2.0). Aligned reads were annotated and quantified with FeatureCounts (v.2.0.0). Gene expression levels were compared in R (v. 3.6) using edfeR package (v.3.268). Samples were normalized by Trimmed Mean of M-values (TMM) method and expression levels were expressed as Counts Per Million (CPM).

RT-qPCR validation of RNAseq data

Several genes were selected for real-time qPCR validation of RNAseq data (Suppl. Table S7). Genes which exhibited the highest up- or down-regulation in one of the comparisons were selected for validation. The expression of those genes was quantified in more than one sample to perform multiple comparisons. OligoPerfect Primer Designer (Thermo Fisher) online tool was used for primer design. RT-qPCR was performed according to (Olguín et al., 2009). Four constitutive genes (Margalef-Català et al., 2016a) were evaluated as internal controls. From those, *gyrA* and *gyrB*, which presented the less variation between samples, were selected. The correlation between the RNAseq data and those values obtained with the RT-qPCR was good with a R^2 = 0.9 (Suppl. Figure S8).

Proteomic analyses

For proteomic analyses, 30 µg of protein were reduced with 4 mM 1.4-Dithiothreitol (DTT) and alquilated with 8 mM iodoacetamide (IAA) before enzymatic digestion using sequencing-grade Trypsin/Lys-C (ThermoFisher Scientific, CA, USA) at enzyme:protein ratio of 1:50. After digestion, the TMT 10-plex labelling (ThermoFisher Scientific, CA, USA) was performed according to manufacturer instructions. To normalize all samples in the study we included in each TMT batch a pool of all samples labelled with TMT-126 tag. Then, TMT labelled peptides were fractionated by Off-gel (Agilent Technologies) according to manufacturer's protocol. Peptides from the 6 fractions obtained were further separated onto a C18 reversed phase (RP) nano-column (75µm I.D.; 15cm length; 3µm particle diameter, Nikkyo Technos Co. LTD, Japan) on an EASY-II nanoLC from Thermo Fisher. The chromatographic separation was performed with a 90 min gradient using Milli-Q water and acetonitrile (0.1 % formic acid) as mobile phase at a flow rate of 300 nL/min.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro from Thermo Fisher by an enhanced FT-resolution MS spectrum (R = 30,000 FHMW) followed by a data dependent FT-MS/MS acquisition (R = 15,000 FHMW, 40 % NCE HCD) from the most intense ten parent ions with a charge state rejection of one and dynamic exclusion of 0.5 min. Protein identification/quantification was performed on Proteome Discoverer software v.1.4.0.288 (ThermoFisher Scientific) by Multidimensional Protein Identification Technology (MudPIT) combining the 6 raw data files obtained (fractions) for each sample. For protein identification, all MS and MS/MS spectra were analysed using Mascot search engine (v.2.5). The workflow was set up using five different Mascot node combing *Oenococcus oeni* PSU-1 database (1682 entries), *Saccharomyces cerevisiae* database (6049 entries), *Torulaspora delbrueckii* (5025 entries), *Metschnikowia pulcherrima* (15 entries) and contaminants database (247 entries). Two missed cleavages were allowed and an error of 0.02 Da for FT-MS/MS fragmentation mass and 10.0 ppm for a FT-MS parent ion mass were allowed. TMT-10plex was set as quantification modification and oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modifications. The false discovery

rate (FDR) and protein probabilities were calculated by Percolator. For protein quantification, the ratios between each TMT-label against 126-TMT label were used and quantification results were normalized based on protein median.

Statistical analyses

The transcriptomic CPM values (fold change) were analysed using the quasi-likelihood F-test of edgeR package and statistical comparisons were performed with the Benjamini-Hochberg method using p-value cutoff <0.05 with passed FDR <0.05. Statistical analyses for proteomics were performed with Mass Profiler Professional Software (Agilent). Raw data were normalized by Log2 transformation and mean-centered for univariate (Student t-test) $p < 0.05$ was fixed as significant.

Next-Generation Clustered Heat Map (NG-CHM) Builder (Ryan et al., 2020) with hierarchical clustering using the Euclidean distance metric with the ward agglomeration method was used for the analyses of the Differentially Expressed Genes (DEG) and Proteins (DEP) of some selected Cluster Orthologous Groups (COG).

Results and discussion

Fermentation dynamics

Wines fermented with *S. cerevisiae* as sole starter were the quickest AF (7 days) comparing to the sequential inoculations (Figure 28). As it is described in the literature, when more than one species is fermenting, the duration of AF is usually extended (Martín-García et al., 2020). Still, the viability of *T. delbrueckii* and *M. pulcherrima* decreased rapidly when *S. cerevisiae* was inoculated. None non-*Saccharomyces* was detected at the end of the AF (Figure 28).

After filtering the wines, *O. oeni* PSU-1 was inoculated. In all wines *O. oeni* finished the MLF (< 0.1 g/L of L-malic acid). Fermentation performance of *O. oeni* is the result of the interactions with the fermenting yeasts and its molecular adaptation mechanisms to them. In this sense, the interactions will depend on the fermenting yeast species and their compatibility. Under the studied conditions, MLF finished in 8 days (Sc wine), 4 days (Td wine) and 2 days (Mp wine) (Figure 28). The duration of MLF was reduced by the use of non-*Saccharomyces* to the half in Td wine or a quarter

in Mp wine regarding to Sc control wine. This reduction of MLF duration in non-*Saccharomyces* fermented wines has been recently addressed in some other works focused in these yeast – bacteria interactions (**Chapter I: 1, 2**; Ferrando et al., 2020; Martín-García et al., 2020). According to the viability of the bacterium increased from around 2 x 10^7 CFU/mL to 10^8 CFU/mL and gradually decreased to the initial concentration over time (Figure 28). In the case of non-*Saccharomyces* wines, as the time was shortened, the viability at the end of MLF was higher than in Sc wine.

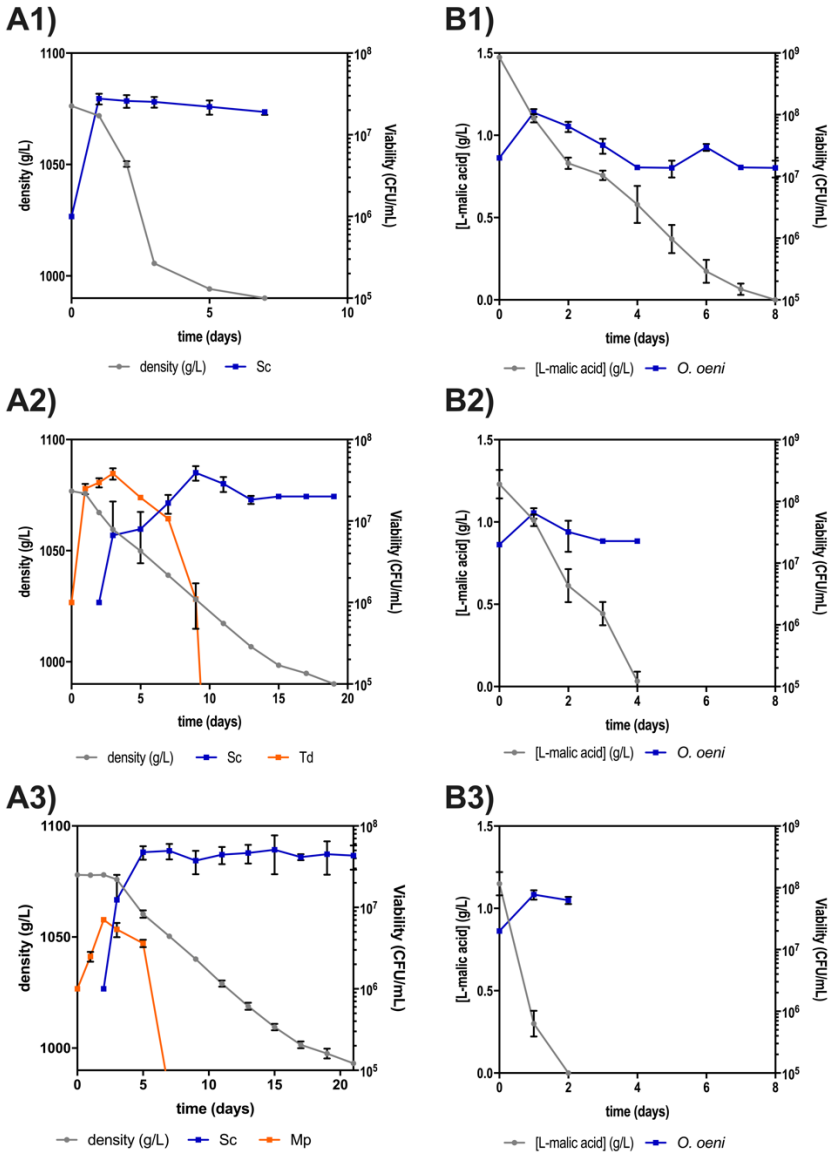


Figure 28. Alcoholic (AF) and malolactic (MLF) fermentation dynamics where density and L-malic acid decrease, respectively are shown together with the viability of the fermenting agents. A1) AF of *S. cerevisiae*. A2) *T. delbrueckii* sequential AF. A3) *M. pulcherrima* sequential AF. B1) MLF in *S. cerevisiae* wine. B2) MLF in *T. delbrueckii* sequential AF. B3) MLF in *M. pulcherrima* sequential AF. Sc, Td, Mp refer to *S. cerevisiae*, *T. delbrueckii*, and *M. pulcherrima*, respectively. Values shown are the mean of triplicates ± SD.

No relevant differences were observed in oenological parameters (Suppl. Table S8). Besides, citric acid was consumed during MLF by *O. oeni* in all conditions, especially in non-*Saccharomyces* fermented wines. Moreover, slightly increased concentration of NOPA was quantified in non-*Saccharomyces* wines as reported in Martín-García et al. (2020).

Global analysis of functions affected by the use of different yeast species

In the RNAseq 1701 expressed sequence tags (EST) were detected in the adaptation to wine during MLF (t_0 and t_f samples) by *O. oeni*. From all, 66 (Sc wine), 69 (Td wine), 101 (Mp wine) were classified as Differentially Expressed Genes (DEG) according to t-Student test MLF t_f vs MLF t_0 (FC > 2, $p < 0.05$) (Figure 29, Suppl. Table S9). These total numbers exclude the DEG corresponding to tRNAs: 13 for Sc wine, 32 for Td wine and 27 for Mp wine, which will be discussed later.

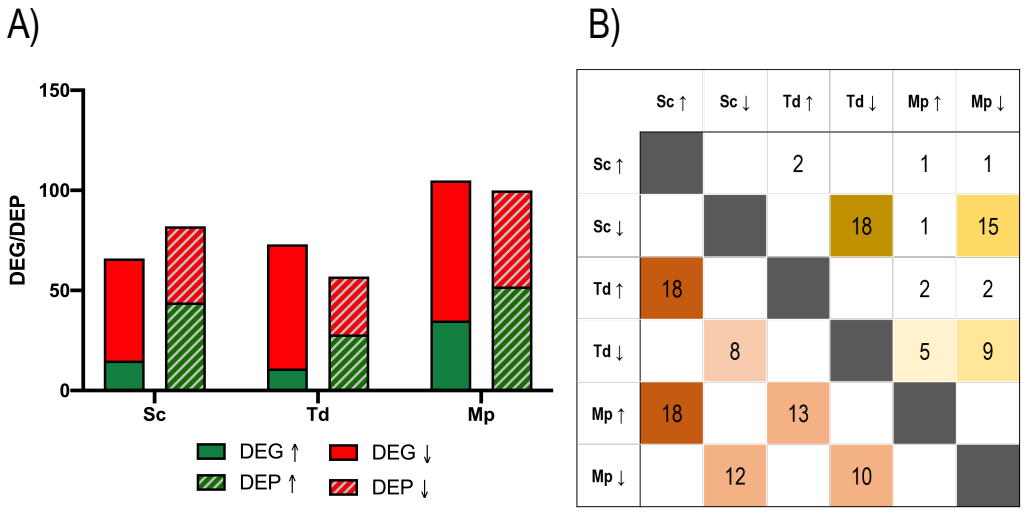


Figure 29. Total differentially expressed genes (DEG) and proteins (DEP) of *O. oeni* PSU-1 during its adaptation in the studied wines. A) Distribution of DEP and DEG of *O. oeni* in each wine with increased (↑) or decreased (↓) regulation or abundance, respectively. B) Table of coincident DEG (upper-right corner) and DEP (down-left corner) with increased (↑) or decreased (↓) regulation/abundance of *O. oeni* in each condition. Cell shadow intensity correspond to increasing number of DEG/DEP for each comparison. Sc, Td, Mp refer to the wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation, respectively. Locus tags corresponding to tRNAs were excluded from this representation.

The number of DEG detected here (Figure 29, Suppl. Table S9) was quite modest regarding to other studies of *O. oeni* wine adaptation mechanisms (Margalef-Català et al., 2016a). Indeed, we are comparing molecular changes of the bacterium in different wines where the only difference is the fermenting yeast. Nevertheless, *O. oeni* functions were more differently expressed fermenting in Mp wine. From those DEGs, the most shared ESTs were related to those DEGs down regulated (Figure 29). Sc wine presented 18-shared down-regulated DEGs with Td wine, and 15 with Mp wine. Besides, only nine were common for Td and Mp wines. In contrast, less homogeneity was observed in the up-regulated DEG for the tested comparisons.

These DEGs were classified in Cluster Orthologous Groups (COGs) in other to better understand the main biological processes affected by the use of non-*Saccharomyces* (Figure 30). Globally, the most DEGs corresponded to the poorly characterized COGs, being the uncharacterized proteins those whose gene expression varied the most. Apart from those DEGs, the COG that clustered the most DEGs was transcription. *O. oeni* behaved similarly in the three wines, in up or down regulation with little difference in down regulation in Mp wine. Similar response for all wines was also observed with carbohydrate transport and metabolism. Cellular process and signalling related COGs did not present many DEGs, especially in the case of *T. delbrueckii* wines. Interestingly, amino acid transport and metabolism related DEGs were down regulated in non-*Saccharomyces* wines, whereas in Sc wine were up regulated. According to nucleotide transport and metabolism, dramatic up regulation was observed in Td wines regarding to the little down regulation in Sc wine and up regulation in Mp wine.

After proteomic analysis, 741 proteins were identified. From all, 658 proteins (present in more than 70 % of the samples in at least one of all conditions) were considered for the analysis. An unpaired Student's T-Test MLF t_f vs MLF t_0 (FC > 1.5, $p < 0.05$) was performed after $\log_2$ normalization resulting in 82 Differentially Expressed Proteins (DEPs) in Sc wine, 57 in Td wine and 100 in Mp wine (Figure 29, Suppl. Table S10). Overall, the number of DEPs that presented an increased abundance was similar to those DEPs with decreased abundance.

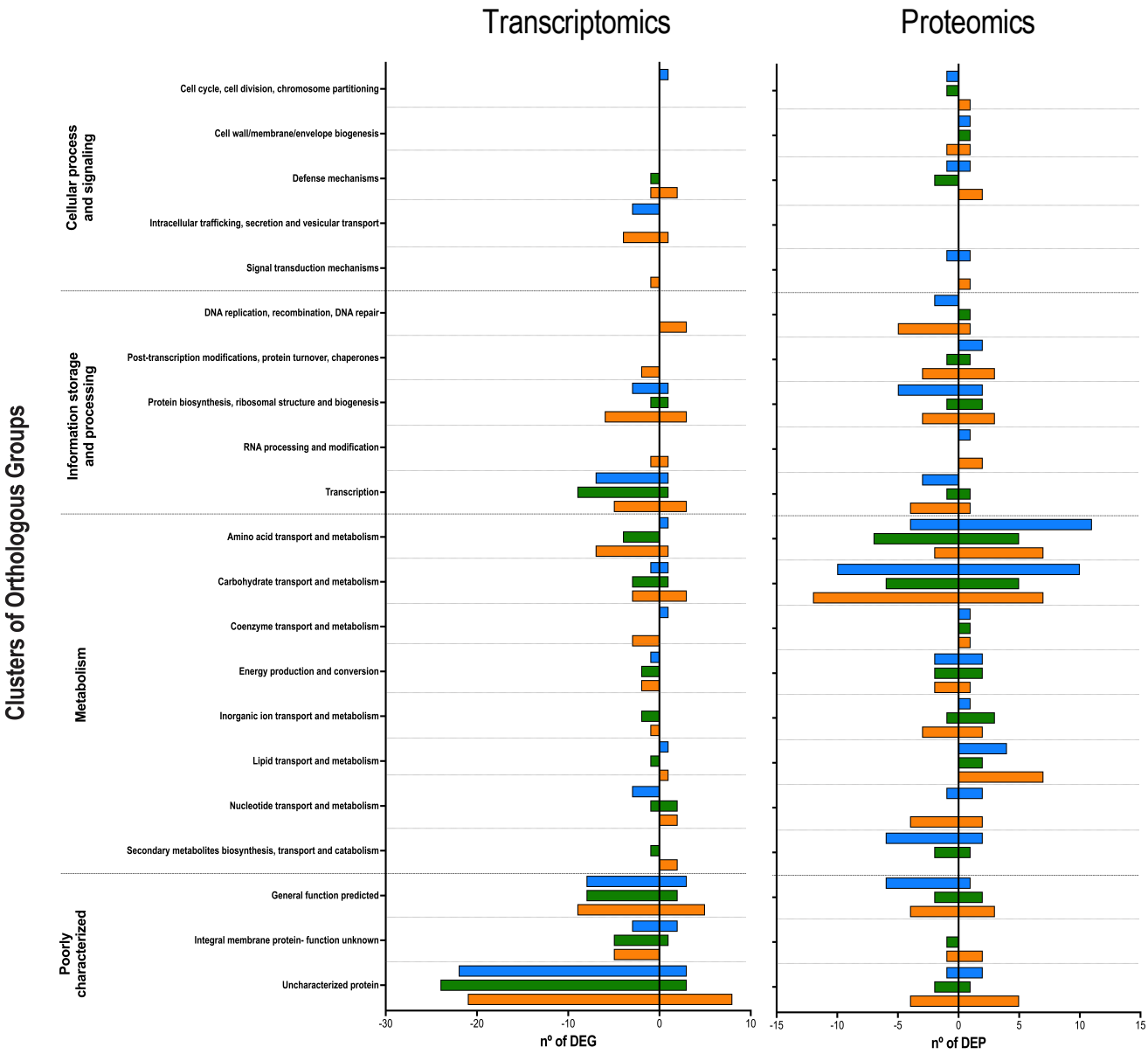


Figure 30. Distribution of differentially expressed genes (DEG) and proteins (DEP) in *O. oeni* fermenting in the three different wines organized in Cluster of Orthologous Groups (COGs). Positive bars represent the number of increased regulation/abundance of DEG/DEP. Negative bars represent the number of decreased regulation/abundance of DEG/DEP. Absence of bar means no DEG/DEP for that COG. Blue, green and orange refer to the wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation, respectively.

Protein study by proteomics presented higher shared DEPs in *O. oeni* adaptation for the three tested wines than DEGs (Figure 29). As we observed in DEG, the highest number of shared DEP were found when comparing Td or Mp wines with Sc wine. 18 DEPs with increased abundance of Td and Mp wines were common to Sc and, from them, 13 were shared between Td and Mp wines. Similar behaviour was observed with DEPs with decreased abundance (Figure 29).

Analogously to DEGs, DEPs were classified into COGs (Figure 30). It is worth to point that little number of DEPs corresponded to poorly characterized COGs. In this sense, the most variable COGs were related to metabolism, being amino acid and carbohydrate transport and metabolism related the ones that clustered the most DEPs. In this way, a homogeneous response was observed in amino acid transport and metabolism COG with the exception of less abundant DEPs in Mp wine. Indeed, changes in these two COGs are related with adaptation in wine related conditions (Margalef-Català et al., 2016a). Also, *O. oeni* proteome was enriched by lipid transport and metabolism proteins with an increased abundance.

Main metabolisms modified by the use of different fermenting yeasts

Carbohydrate transport and metabolism

Regarding the carbohydrate metabolism, *O. oeni* in Td wines was the one showing less changes in the proteome along MLF. The two DEPs with the highest increase in abundance in *O. oeni* from Mp and Sc wines, but not DEP from Td wine, were 2-hydroxyisocaproate dehydrogenase (OEOE_RS05695) and phosphoketolase (OEOE_RS05700) (Suppl. Table S10). These two proteins gathered in the same cluster due to the high values of differential expression compared to the other DEP (Cluster I, Figure 31A). In Sc wines, *O. oeni* increased the abundance of these two proteins in more than 10-fold at the end of MLF, being the most DEP among all detected proteins by far. The enzyme 2-hydroxyisocaproate dehydrogenase (HicD) is associated to the production of 2-hydroxyisocaproic acid (HICA) from leucine. This compound has antifungal activity (Axel et al., 2016) and its production by *Lactobacillus* and *Leuconostoc* species has been described in different fermented foods (Axel et al., 2016; Park et al., 2017). There are no previous reports about HicA or HICA in *O. oeni*. The role in microbial interaction of this compound may be worth of future consideration in MLF research. Concerning phosphoketolase (PK), this is a key enzyme in the heterolactic fermentation of sugars, named the phosphoketolase pathway. This protein catalyzes the cleavage of D-xylulose 5-phospate and inorganic phosphate to form acetyl phosphate and D-glyceraldehyde 3-phosphate. Although residual sugars were barely consumed at the end of MLF (Suppl. Table S8) some authors have described that most of the ATP formed from the phosphoketolase pathway in the presence of

ethanol occurs once L-malic acid has been consumed (Contreras et al., 2018), as it occurs with other alternative energy sources like citric acid (Bartowsky and Henschke, 2004) or mannoproteins (**Chapter III: 2**). The increase in the abundance of PK at the end of MLF could be indicative of the metabolic changes suffered by *O. oeni* in response to L-malic acid exhaustion. The increase in PK synthesis would prepare the cell for the consumption of other energy sources, sugars in this case. However, this was only detected in *O. oeni* from Mp and Sc wines, not from Td wines, where the activation of PK may have occurred later.

The cluster II (Figure 31A) included mainly proteins of the central pyruvate metabolism (i.e., pyruvate oxidase and enolase) that increased their abundance along MLF in the different wines. It is worthily to note that all the DEPs in Td wines of this cluster are coincident in Sc wines, meaning that *O. oeni* had a more similar behavior for these proteins in Td and Sc wines. However, other DEP were detected exclusively in *O. oeni* from Sc or Mp wines. The increased abundance of these proteins at the end of MLF may be associated to the metabolic switch in response to L-malic acid exhaustion, as hypothesized for PK.

The proteins that decreased their abundance were grouped in cluster III (Figure 31A). The function of the proteins included in this cluster was diverse, including some sugar transporters and glycosyl transferases associated to exopolysaccharide synthesis. The downregulation of genes encoding for many of these proteins was previously described by Margalef et al. (2016) in response to wine conditions (OEOE_RS06015, OEOE_RS01620, OEOE_RS02240, OEOE_RS07345, OEOE_RS07030, OEOE_RS07030, OEOE_RS07015, OEOE_RS02510, OEOE_RS07050, OEOE_RS07045, OEOE_RS01210; Suppl. Table S10). Interestingly, most of the DEP included in cluster III showed a different behavior depending on the yeast strain/s inoculated in the must. Only two DEPs were coincident in all the conditions: acetoin reductase (OEOE_RS07730) and one aldehyde-alcohol dehydrogenase (OEOE_RS06015). The inhibition of these two proteins in response to wine conditions has been already described by Margalef et al. (2016).

The transcriptomic analyses detected less changes than the proteomic analysis. The detected DEGs associated to carbohydrate metabolism showed some coincidences

with the DEPs of this functional category. The changes in gene expression showed mostly down-regulated genes (Supp. Table S9, Figure 32A), some of them related to sugar transport and exopolysaccharide synthesis, as seen in the proteomic analysis. The specific behaviour depending on the yeast/s strain inoculated is also evident in the DEGs profiles, in which all the genes are differentially expressed exclusively in one of the three conditions.

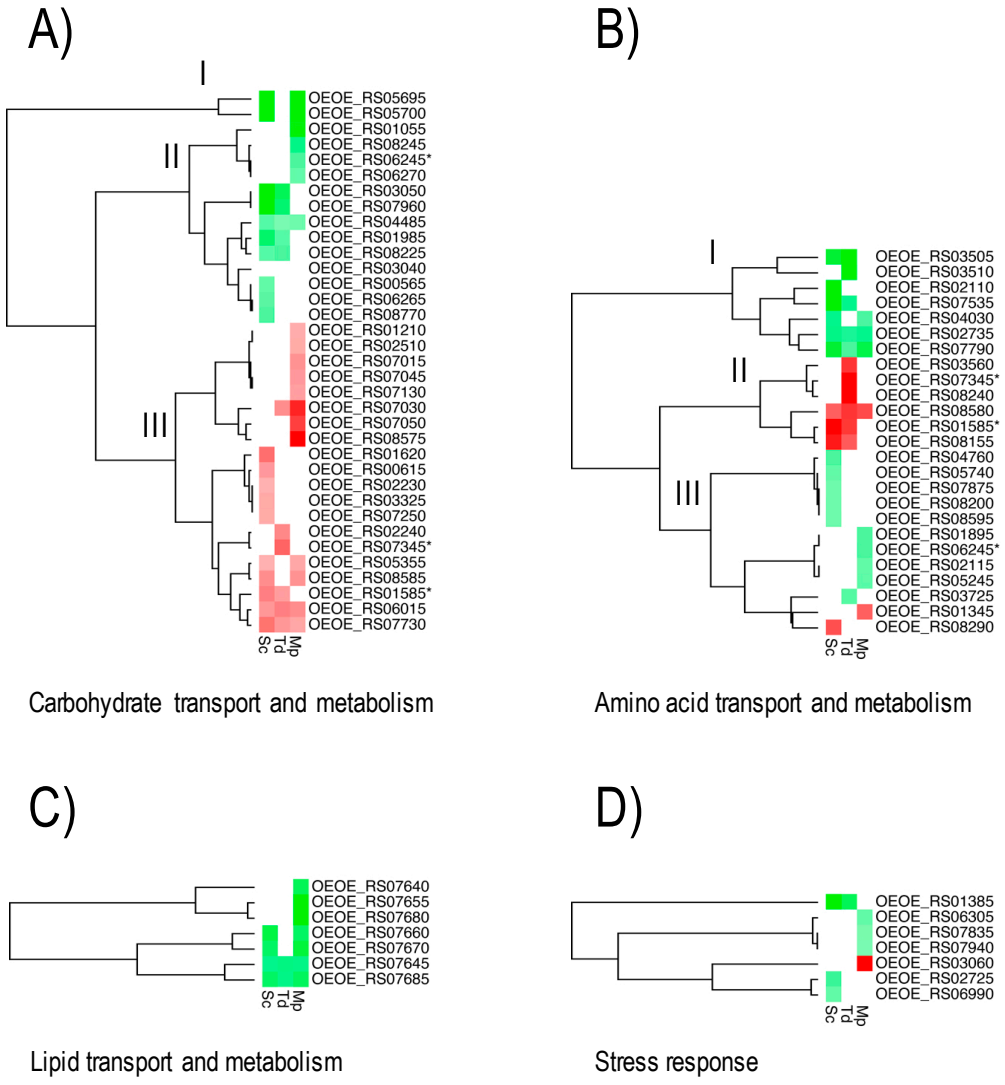


Figure 31. Heat map and clusterization of some differentially expressed proteins (DEP) in *O. oeni* PSU-1 fermenting in the three different wines. A) DEP of carbohydrate transport and metabolism Cluster of Orthologous Group (COG). B) DEP of amino acid transport and metabolism COG. C) DEP of lipid transport and metabolism COG. D) DEP of some stress response related locus tags. Sc, Td and Mp refer to the wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation, respectively. Total length of the dendrogram represent 100% of similarity. Green (increased) and red (decreased) intensity represent increasing fold change for each DEP. No colour means no DEP. Colour intensity is relative to each heatmap. *locus tag classified in more than one COG.

Amino acid transport and metabolism

The changes in the proteome observed in *O. oeni* related to amino acid transport and metabolism showed that 11 out of the 17 DEPs with increased abundance were related to peptidase activity and peptide transport (Figure 31B, Suppl. Table S10). Cluster I (Figure 31B) included mostly proteins related to peptide transport (OEOE_RS02110, OEOE_RS03505, OEOE_RS03510, OEOE_RS04030; Suppl. Table S10). It also included a dipeptidase (OEOE_RS02735) that was previously reported as differentially increased in response to wine conditions (Margalef et al., 2016). Remarkably, this peptidase and one ABC-type peptide transport protein (OEOE_RS07790) had increased their abundance in *O. oeni* within the three wines. These two proteins showed a common behaviour in all the conditions. However, most of the *O. oeni* DEPs related to amino acid metabolism behaved differently depending on the yeast strain/s used in AF. In fact, cluster III includes several peptidases differentially expressed only in *O. oeni* from Sc wines (OEOE_RS08595, OEOE_RS08200; Suppl. Table S10). Additionally, glutathione reductase was one of the proteins with increased abundance only in Sc wine. This protein has been previously related to stress response and adaptation to wine conditions (Cecconi et al., 2009; Margalef-Català et al., 2016a; Silveira et al., 2004). Cluster III also grouped some DEP only detected in *O. oeni* from Mp wines and one from Td wine. These differences in the *O. oeni* proteomic profile indicates that the nitrogen composition in wines after AF, which depends on the yeast strain/s metabolism, may have greatly influenced *O. oeni* peptide utilization. According to Ritt et al. (2008) the level of biosynthesis of *O. oeni* peptidases depends on the peptides present in medium. The same authors reported that the peptides from yeast decreased the levels of peptidase activity and down regulated peptidase gene transcription. In this study, *O. oeni* from Sc wine showed the highest number of positively DEPs with peptidase function. This could be related to the lower availability of peptides at the end of MLF in Sc wine with respect to the wines inoculated with non-*Saccharomyces*. The double inoculated yeast population in Td and Mp wines, also inoculated with *S. cerevisiae*, might account for the higher concentration of peptides at the end of MLF. However, the relationship between peptide composition and *O. oeni* peptidase activity requires further investigation.

Altogether, the results highlight the relevance of wine nitrogen composition and the ability of *O. oeni* to adapt to its environment. Liu et al. (2010) reported the essential role of peptidases for bacterial growth or survival as they are encoded in all LAB genomes. Not in vain, in the *O. oeni* PSU-1 genome are described more than 20 peptidases.

Cluster II (Figure 31B) included the proteins that decreased their abundance. Interestingly 5 out of the 6 DEPs of the cluster were identified as threonine dehydrogenase like proteins. The down regulation of three of them (OEOE_RS08580, OEOE_RS08240, OEOE_RS03560; Suppl. Table S10) have been previously reported in response to wine conditions (Margalef et al., 2016). The precise function of these proteins is not clear enough to discuss their possible role. Actually, some of these enzymes were identified generically as alcohol dehydrogenases in former versions of the PSU-1 genome annotation.

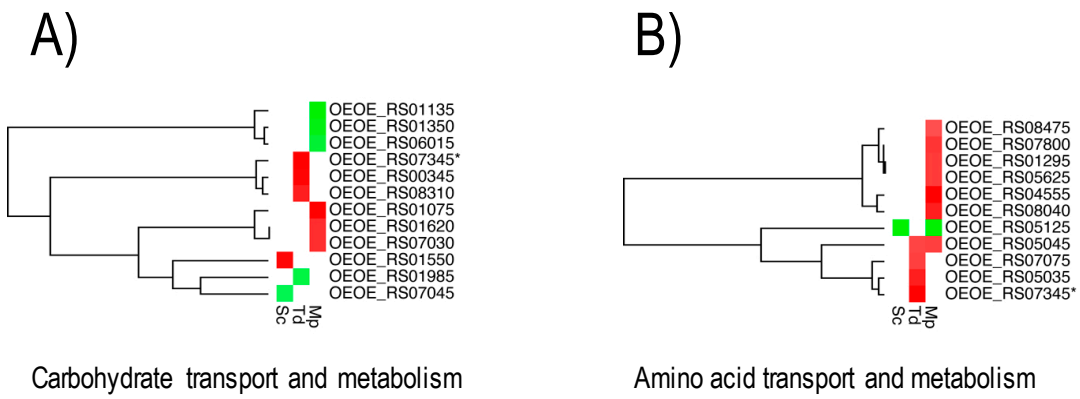


Figure 32. Heat map and clusterization of some differentially expressed genes (DEG) in *O. oeni* PSU-1 fermenting in the three different wines. A) DEG of carbohydrate transport and metabolism Cluster of Orthologous Group (COG). B) DEG of amino acid transport and metabolism COG. Sc, Td and Mp refer to the wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation, respectively. Total length of the dendrogram represent 100% of similarity. Green (increased) and red (decreased) intensity represent increasing fold change for each DEG. No colour means no DEG. Colour intensity is relative to each heatmap. *locus tag classified in more than one COG.

The transcriptional analyses of the amino acid metabolism showed mostly down-regulated genes in *O. oeni* from Td and Mp wines (Figure 32B). The predominant function among these proteins was related to amino acid and peptide transport but different DEGs were detected depending on the wine. The only up-regulated gene was a carboxypeptidase of *O. oeni* from Sc wine (Suppl. Table S9). Again, the results seem

to point to a different regulation of the nitrogen utilization in *O. oeni* that would be conditioned by the nitrogen composition of wine after AF, consequence of the yeast strain/s metabolism.

Lipid transport and metabolism

Concerning the lipid metabolism, all the DEPs showed an increase in their abundance (Figure 31C). *O. oeni* from Mp wine showed the highest number of proteins (7 in total) responding to wine conditions in this metabolic category. All the DEPs were identified with functions related to fatty acids (FA) biosynthesis (i.e., acyl and carboxyl transfer and metabolism). The DEPs detected in *O. oeni* from Sc and Td wines were coincident with those detected in Mp wine. This would mean that the activation of FA biosynthesis is a common mechanism in *O. oeni* although the response, in terms of number of DEP, was stronger in Mp wines than in Sc and Td wines. No coincidences were found in this case with the transcriptomic response. This lack of correspondence is a usual phenomenon since transcriptional regulation might be different from post-transcriptional mechanisms than influence protein translation.

Changes in FA composition to adapt to wine stress factors, such as ethanol and low pH, has been described in *O. oeni* (Grandvalet et al., 2008). The different DEPs profile observed in *O. oeni* may be related to differences in wine composition after AF due to specific yeast strain's metabolism. The observed increased abundance of proteins associated to FA biosynthesis and the maintenance of other proteins related to this function (no decreases in protein concentration were detected) stands out the key role of the changes in the membrane to adapt to stress conditions.

Stress related mechanisms

Some DEPs associated to described *O. oeni* stress response mechanism could be identified (Figure 31D; Suppl. Table S10). *O. oeni* from Mp and Sc showed the increase in the protein abundance of three stress related proteins, although there was no coincidence among them. *O. oeni* from Sc wine showed an increase in concentration of one ATPase (OEOE_RS03185), ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones (OEOE_RS02725), dTDP-glucose 4,6-dehydratase (OEOE_RS06990) and the heat shock protein Hsp20 (OEOE_RS01385). The increase

Hsp20 was also detected in Td wine. In the case of Mp wine, *O. oeni* showed an increase in abundance of D-alanine-D-alanine ligase (OEOE_RS03220), DnaJ chaperone (OEOE_RS06305), the universal stress protein UspA (OEOE_RS07940), and thioredoxin (OEOE_RS07835). These all proteins have been previously associated with the stress response to wine conditions (Beltramo et al., 2006; Margalef-Català et al., 2016a; Olguín et al., 2010; Yang et al., 2020). The differences observed in *O. oeni* among the three wines were detected in a small number of DEP, meaning that the general stress response must have been quite similar disregarding the yeast strain/s responsible for AF. Notwithstanding, the stress protein. Hsp20 resulted indicative of the adaptation to more difficult wine conditions. This heat shock protein showed a higher increase in the wines showing a longer MLF. Hsp20 was 4-fold augmented in Sc wine, 3-fold in Td wine and no changes were detected in Mp wine, following the correlation of a higher increase in protein abundance with a slower MLF. This heat shock protein, also known as Lo18 or Hsp18, has long been described as one of the main stress responding proteins in *O. oeni* with chaperone and membrane protection functions (Coucheney et al., 2005b; Guzzo et al., 1997). Indeed, Hsp20 has also been proposed as a stress response marker (Coucheney et al., 2005a; Olguín et al., 2010).

Differential expression of tRNAs

Some DEGs related to tRNAs were detected within the samples (Figure 33). Few information about the impact of the regulation of tRNAs in MLF performance is available in the literature. Overall, tRNAs are related with higher protein biosynthesis and, thus, with higher metabolic activity (Raina and Ibba, 2014) that can be related with fast fermentations. In this sense, *O. oeni* strains with higher metabolic activity exhibit an upregulation in tRNAs (Sternes et al., 2017). Besides, no information is available in terms of adaptation of a specific strain.

The main changes were observed in Td and Mp wines. Td wines presented a general down-regulation in tRNAs when comparing the state of *O. oeni* at the end of MLF and the initial stage of the fermentation. In Mp wine, the observation was the contrary. Low differentially expressed tRNAs were detected in Sc wine. In the present study MLF performance in Mp wine was the fastest and also, the condition which presented an up-regulation in tRNA expression. Regarding to Sc and Td wines, the presented

correlation does not fit with the tRNA regulation. In this sense, more information about tRNA biological implication should be needed to better understand the observed changes.

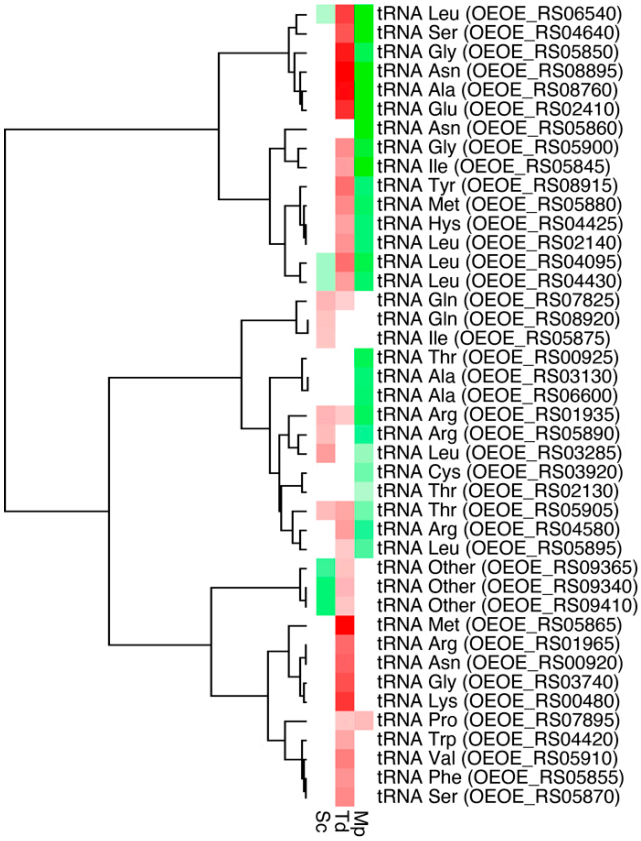


Figure 33. Heat map and clusterization of the differentially expressed genes (DEG) corresponding to tRNA locus tags in *O. oeni* PSU-1 fermenting in the three different wines. Sc, Td and Mp refer to the wines *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation, respectively. Total length of the dendrogram represent 100% of similarity. Green (increased) and red (decreased) intensity represent increasing fold change for each DEG. No colour means no DEG.

Other modified metabolisms

In addition to the already highlighted metabolisms, some other functional categories were affected along MLF. The gene expression of many transcriptional regulators was down-regulated (Suppl. Table S9). The inhibition of some ribosomal proteins was also detected both in the proteomic and transcriptomic analyses (Suppl. Tables 3 and 4). Once more, the response was very dependent on the yeast strain/s developing AF.

Regarding the L-malic acid consumption, the malolactic enzyme (OEOE_RS07545) showed a similar increase in the abundance (2-3-fold) in the three studied wines (Suppl. Table S10), therefore it was not possible to correlate the increase of this enzyme with the different MLF velocity.

Conclusion

The specific DEGs and DEPs detected in *O. oeni* PSU-1 depending on the yeast strain/s used in the AF reflects that wine composition, result of each yeast metabolic traits, greatly influences *O. oeni* molecular mechanisms of adaptation to wine. Non-*Saccharomyces* yeast promoted a faster MLF. The abundance of malolactic enzyme was similar in all the conditions and would not explain the differences in MLF duration. The expression of the stress protein Hsp20 was confirmed as a reliable marker of stressful conditions and its increase could be correlated to slower MLF. Among the complex *O. oeni* molecular response, depending on the fermenting yeast, the specificity of peptide utilization might have played a key positive role in *O. oeni* adaptation to wine in Mp and Td wines. This fact encourages further research on the characterization of peptides released by different yeast species and their use by *O. oeni* in order to better understand microbial interactions in wine and their effect on MLF.

Acknowledgments

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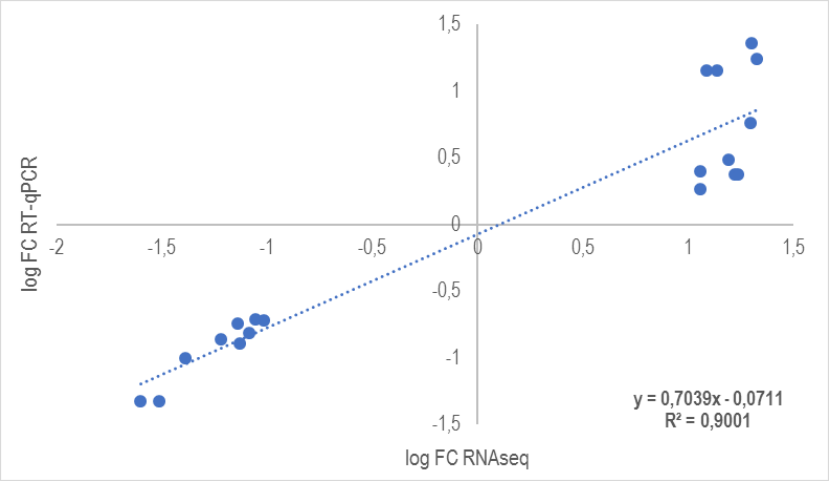
Supplementary Figures

Supl. Figure S8. Correlation of RNAseq data and RT-qPCR of selected genes of Suppl.Table S7.



Supplementary Tables

Supl. Table S7. List of primers used to validate the RNAseq data by RT-qPCR

Locus tag and gene symbol	Sequence (5'→3')	Amplicon length (bp)	Reference
OEOE_RS00015 (OEOE_0003) (*yaaA*)	F- CGGCGGCAGTGCAAAATTTT R- GGTCTCCTGGATAGAGTTTCCG	101	This work
OEOE_RS01570 (OEOE_0328) (*pdhA*)	F- GCAAAGGGCCGGTTTTGATT R- GCGCATGCGAATCAAAGGAT	137	This work
OEOE_RS02115 (OEOE_0439)	F- GCATGCCGGCGATAAACAAT R- TGCTGGCCACCGGATAATTT	102	This work
OEOE_RS02240 (OEOE_0466)	F- ACGATGTGGCCATTCAAGGT R- CACTAAGAGCCAGGGTTGCA	126	This work
OEOE_RS02715 (OEOE_0570) (*clpP*)	F- CGGTACCAAAGGCAAGCGTTTTAT R- CTCTTCCGAGTCTTCAAAAGTTGAT	131	Deltramo et al. (2006)
OEOE_RS03505 (OEOE_0734)	F- GCTATCGACTCGGCGAGAAA R- GTCGCTTCTGCTACTCTGCA	149	This work
OEOE_RS03800 (OEOE_0793)	F- TTGGCGGAAATAATGCAGCG R- CATGAAAAGCCGAGGATGCC	217	This work
OEOE_RS04045 (OEOE_0841) (*oppA*)	F- GGAAGCTGGTCGAACAAGGA R- AGTGTCGCTGCATTTACCCA	161	This work
OEOE_RS04565 (OEOE_0952) (*glnA*)	F- AATGGAAACGGCATGCACAC R- CAAAACCAGGAGTCAAGCGC	193	This work

OEOE_RS06015 (OEOE_1248) (*adhE*)	F- GCCCATAAAACCGGTGGAGA R- GCGCGCAATATCGGCATAAT	165	This work
OEOE_RS07550 (OEOE_1565) (*mleR*)	F- GCCCACGAAGAAATTGACCG R- TGGAGCCGGCTTCATTAGTC	148	This work
OEOE_RS07670 (OEOE_1590) (*fabD*)	F- CAAAGCGGGGACAAACGTTT R- CCAAAGCATCACCAGCAACC	166	This work
OEOE_RS07675 (OEOE_1591) (*fabK*)	F- TCCAGTTGTTCCTTCGACCG R- CGGCAGCAATAACCGGAATG	164	This work
OEOE_RS07690 (OEOE_1594)	F- TGCTCGACAGTTGAGGCTTT R- CTCGGTCATTTGTCGGTGGA	181	This work
OEOE_RS07730 (OEOE_1602)	F- AACACCGGCAGACCAAGTAG R- TCTTTTGCTGCCGCTTGAAC	216	This work
OEOE_RS01985 (OEOE_0413) (*ldhD*)	F- GCCGCAGTAAAGAACTTGATG R- TGCCGACAACACCAACTGTTT	102	Desroche et al. (2005)
OEOE_RS04805 (OEOE_1000) (dpolIII)	F- AATTCGCACGGATTGTTTTC R- GCGAACCAGCATAGGTCAAT	103	Stefanelli (2014)
OEOE_RS04780 (OEOE_0995) (*dnaG*)	F- TGTGGACGGAGTGGCAATGT R- CAGTATTTTCTGTATATTTACTATCG	127	Desroche et al. (2005)
OEOE_ RS00030 (OEOE_0006) (*gyrA*)	F- CGCCCGACAAACCGCATAAA R- CAAGGACTCATAGATTGCCGAA	95	Desroche et al. (2005)
OEOE_RS00025 (OEOE_0005) (*gyrB*)	F- GAGGATGTCCGAGAAGGAATTA R- ACCTGCTGGGCATCTGTATTG	107	Desroche et al. (2005)

Supl. Table S8. Oenological parameters of wines after alcoholic (AF) and malolactic (MLF) fermentations. Values shown are the means of triplicates ± SD. Statistics were calculated independently for each grape variety. *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* correspond to *S. cerevisiae, T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively.

	Must	*S. cerevisiae*		*T. delbrueckii*		*M. pulcherrima*	
		AF	MLF	AF	MLF	AF	MLF
Glucose + fructose (g/L)	-	0.33 ± 0.08^{a}	0.31 ± 0.09^{a}	0.92 ± 0.08^{c}	0.76 ± 0.1^{bc}	0.5 ± 0.17^{ab}	0.44 ± 0.12^{a}
Malic acid (g/L)	2.09	1.47 ± 0.02^{c}	n.d.^{a}	1.23 ± 0.09^{b}	n.d.^{a}	1.18 ± 0.07^{b}	n.d.^{a}
Citric acid (g/L)	0.32	0.29 ± 0^{d}	0.11 ± 0.01^{c}	0.3 ± 0.01^{d}	0.07 ± 0.03^{b}	0.27 ± 0.01^{d}	n.d.^{a}
Acetic acid (g/L)	0.05	0.21 ± 0.01^{b}	0.27 ± 0.01^{c}	0.14 ± 0.01^{a}	0.19 ± 0^{b}	0.33 ± 0.03^{d}	0.43 ± 0.01^{e}
NOPA (mg/L)	173.5	51.7 ± 3.8^{a}	55.33 ± 6.4^{ab}	67.4 ± 7.2^{b}	67.1 ± 6.2^{b}	51.3 ± 3.6^{a}	58.6 ± 2.6^{ab}
NH_4 (mg/L)	96.4	16.7 ± 3.8^{a}	15.1 ± 4.1^{a}	16.2 ± 3.1^{a}	15 ± 3.5^{a}	14.9 ± 1.3^{a}	14.4 ± 0.9^{a}
pH	3.6	3.45 ± 0.03^{a}	3.78 ± 0.01^{c}	3.47 ± 0.02^{a}	3.61 ± 0.02^{b}	3.57 ± 0.02^{b}	3.82 ± 0.01^{c}
Ethanol (% vol/vol)	-	10.83 ± 0.24^{a}	-	10.76 ± 0.17^{a}	-	10.31 ± 0.85^{a}	-

$^{a–c}$ Values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison test. - not determined. N.d.: not detected.

Supl. Table S9. Relative expression of genes (expressed as log_2FC) affected during malolactic fermentation (MLF) by the use of non-*Saccharomyces*, grouped by Clusters of Orthologous Groups (COGs). Sc, Td and Mp correspond to *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, respectively.

Locus tag		Description	log_2FC		
			Sc	Td	Mp
Cellular process and signaling					
Cell cycle. cell division. chromosome partitioning					
OEOE_RS05535		Cell division protein FtsL	1.04		
Cell wall/membrane/envelope biogenesis					
-					
Defense mechanism					
OEOE_RS00420		Beta-lactamase class C related penicillin binding protein			-1.01
OEOE_RS05465*	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase. EC 6.3.2.6			1.10
OEOE_RS05475*	*purE*	N5-carboxyaminoimidazole ribonucleotide mutase. N5-CAIR mutase. EC 5.4.99.18			1.06
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-1.41	
Intracellular trafficking. secretion. and vesicular transport					
OEOE_RS01945	*secG*	Protein-export membrane protein SecG			1.13
OEOE_RS06045		Type II secretory pathway/competence. pseudopilin	-1.27		-1.22
OEOE_RS06050		Competence protein ComGC	-1.83		-1.99
OEOE_RS06055		Type II secretory pathway/competence component	-1.04		-1.45
OEOE_RS06860	*secE*	Protein translocase subunit secE/sec61 gamma			-1.63
Signal transduction mechanisms					
OEOE_RS09170		Response regulatory domain-containing protein			-1.02

Information storage and processing					
DNA replication. recombination and repair					
OEOE_RS04615		DNA uptake protein-like DNA-binding protein			1.03
OEOE_RS04755	*recO*	DNA repair protein RecO (Recombination protein O)			1.14
OEOE_RS05955	*xseB*	Exodeoxyribonuclease 7 small subunit. EC 3.1.11.6			1.66
Post-translational modification. protein turnover and chaperones					
OEOE_RS06725	*groL*	60 kDa chaperonin (GroEL protein) (Protein Cpn60)			-1.30
OEOE_RS06820		Lipoyl-binding domain-containing protein			-1.13
Protein biosynthesis. ribosomal structure and biogenesis					
OEOE_RS00985		Acetyltransferase. including N-acetylase of ribosomal protein	-1.08		
OEOE_RS02080		LSU ribosomal protein L7AE			1.12
OEOE_RS02145		50S ribosomal protein L28			1.50
OEOE_RS02560		Acetyltransferase. GNAT family			1.38
OEOE_RS02865	*rpsS*	30S ribosomal protein S19			-1.48
OEOE_RS02870	*rplV*	50S ribosomal protein L22			-1.30
OEOE_RS02900	*rplX*	50S ribosomal protein L24			-1.20
OEOE_RS04010		Acetyltransferase. GNAT family	-1.63		
OEOE_RS04155		Acetyltransferase. GNAT family		-1.06	
OEOE_RS06185	*rpsT*	30S ribosomal protein S20		1.37	-1.30
OEOE_RS06475		Acetyltransferase. GNAT family			-1.04
OEOE_RS06745	*rimI*	Acetyltransferase	1.21		
OEOE_RS08350		Cytidine deaminase. EC 3.5.4.5 (Cytidine aminohydrolase)	-1.13		
OEOE_RS08600	*rpmE2*	50S ribosomal protein L31 type B			-1.13
RNA processing and modification					

OEOE_RS03835	*rimM*	Ribosome maturation factor RimM			1.35
OEOE_RS05985	*rnj*	Ribonuclease J. RNase J. EC 3.1.-.-			-1.20
Transcription					
OEOE_RS00285		Transcriptional regulator. HTH and ATP-binding Schlafen-like domain	-1.26		
OEOE_RS00360		HTH merR-type domain-containing protein			-1.08
OEOE_RS00440		Transcriptional regulator			-1.24
OEOE_RS00455		Transcriptional regulator. xre family		-1.04	
OEOE_RS00460		Transcriptional regulator. AraC family	-1.47		
OEOE_RS00930		Transcriptional regulator. xre family	-1.46		
OEOE_RS01065		Transcriptional regulator		-1.08	
OEOE_RS02290		Transcriptional regulator. ArsR family			1.34
OEOE_RS02390		Transcriptional regulator		-1.01	
OEOE_RS02485		Transcriptional regulator. PadR family		1.04	
OEOE_RS02600		Transcriptional regulator	-1.34	-1.22	
OEOE_RS03455		Response regulator of the LytR/AlgR family	-1.31		-1.30
OEOE_RS03500		Transcriptional regulator		-1.07	
OEOE_RS05730	*yxeR*	Transcriptional regulator. Fur family		-1.13	-1.21
OEOE_RS05965	*nusB*	Transcription antitermination protein NusB (Antitermination factor NusB)			1.64
OEOE_RS07600		Transcriptional regulator. AraC family	-1.06		
OEOE_RS07690		Transcriptional regulator. MarR family		-1.02	
OEOE_RS08565		Transcriptional regulator. helix-turn-helix XRE-family	1.17		
OEOE_RS08690		Transcriptional regulator. GntR family			-1.30
OEOE_RS09010		LacI family DNA-binding transcriptional regulator		-1.12	1.32
OEOE_RS09110		LysR family transcriptional regulator	-1.46	-1.71	

Metabolism					
Amino acid transport and metabolism					
OEOE_RS01295		ABC-type antimicrobial peptide transport system. ATPase component			-1.09
OEOE_RS04555	*miaA*	tRNA dimethylallyltransferase. EC 2.5.1.75			-1.46
OEOE_RS05035		Aspartate racemase. EC 5.1.1.13		-1.25	
OEOE_RS05045		ABC-type polar amino acid transport system. ATPase component		-1.04	-1.08
OEOE_RS05125		D-alanyl-D-alanine carboxypeptidase	1.02		1.12
OEOE_RS05625		Amino acid/polyamine/organocation transporter. APC superfamily			-1.09
OEOE_RS07075		Spermidine/putrescine ABC transporter permease protein		-1.06	
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-1.41	
OEOE_RS07800		ABC-type dipeptide/oligopeptide/nickel transport system. permease component			-1.12
OEOE_RS08040		Amino acid ABC transporter ATP-binding protein. PAAT family			-1.25
OEOE_RS08475		Serine acetyltransferase. EC 2.3.1.30			-1.00
Carbohydrate transport and metabolism					
OEOE_RS00345		Arabinose efflux permease		-1.24	
OEOE_RS01075	*araK*	L-ribulokinase (Putative)			-1.27
OEOE_RS01135		Carbonic anhydrase. EC 4.2.1.1			1.46
OEOE_RS01350		Cellobiose-specific PTS system IIC component			1.24
OEOE_RS01550	*aldA*	Lactaldehyde dehydrogenase. EC 1.2.1.22	-1.21		
OEOE_RS01620		Cellobiose-specific PTS system IIB component			-1.04
OEOE_RS01985		Phosphoglycerate dehydrogenase-like dehydrogenase		1.07	
OEOE_RS06015	*adhE*	Aldehyde-alcohol dehydrogenase			1.14
OEOE_RS07030		Carbohydrate ABC transporter ATP-binding protein. CUT1 family			-1.04
OEOE_RS07045		Carbohydrate ABC transporter substrate-binding protein. CUT1 family	1.01		
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-1.41	

OEOE_RS08310		2-isopropylmalate synthase. EC 2.3.3.13		-1.11	
Coenzyme transport and metabolism					
OEOE_RS05765		HAD superfamily phosphatase			-1.06
OEOE_RS07110		6-carboxy-5.6.7.8-tetrahydropterin synthase. EC 4.1.2.50 (Queuosine biosynthesis protein QueD)	1.22		-1.80
Energy production and conversion					
OEOE_RS01570		Pyruvate dehydrogenase E1 component subunit alpha			-1.18
OEOE_RS01575		Acetoin dehydrogenase complex. E1 component. beta subunit			-1.18
OEOE_RS01580		Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	-1.05	-1.05	
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-1.41	
Inorganic ion transport and metabolism					
OEOE_RS03490	*crcB*	Putative fluoride ion transporter CrcB		-1.61	-1.67
OEOE_RS08285		ABC-type metal ion transport system. periplasmic component/surface antigen		-1.38	
Lipid transport and metabolism					
OEOE_RS04670		1-acyl-sn-glycerol-3-phosphate acyltransferase. EC 2.3.1.51			1.03
OEOE_RS04745		Diacylglycerol kinase. EC 2.7.1.107	1.13		
OEOE_RS07660	*fabF*	3-oxoacyl-[acyl-carrier-protein] synthase 2		1.02	
Nucleotide transport and metabolism					
OEOE_RS04460		Diadenosine tetraphosphate (Ap4A) hydrolase related HIT family hydrolase	-1.16		
OEOE_RS05455	*purQ*	Phosphoribosylformylglycinamidine synthase subunit PurQ. FGAM synthase. EC 6.3.5.3	-1.06		
OEOE_RS05460	*purS*	Phosphoribosylformylglycinamidine synthase subunit PurS. FGAM synthase. EC 6.3.5.3	-1.68		
OEOE_RS05465*	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase. EC 6.3.2.6 (SAICAR synthetase)			1.10
OEOE_RS05475*	*purE*	N5-carboxyaminoimidazole ribonucleotide mutase. N5-CAIR mutase. EC 5.4.99.18			1.06
OEOE_RS07755		Nucleoside ABC transporter membrane protein		1.08	
OEOE_RS07760		Nucleoside ABC transporter membrane protein		1.06	
OEOE_RS07770	*rpiA*	Ribose-5-phosphate isomerase A. EC 5.3.1.6 (Phosphoriboisomerase A. PRI)		-1.18	
Secondary metabolites biosynthesis. transport. and catabolism					

OEOE_RS05465*	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase. EC 6.3.2.6 (SAICAR synthetase)			1.10
OEOE_RS05475*	*purE*	N5-carboxyaminoimidazole ribonucleotide mutase. N5-CAIR mutase. EC 5.4.99.18			1.06
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-1.41	
Poorly characterized					
General function predicted					
OEOE_RS00180		Probable membrane transporter protein			1.01
OEOE_RS00275		Alpha/beta superfamily hydrolase	-1.17		-1.39
OEOE_RS00475		Calcineurin-like phosphoesterase		-1.07	-1.06
OEOE_RS00975		ABC-type multidrug transport system. ATPase component	-1.37	-1.34	-1.39
OEOE_RS01330		Aromatic compounds catabolism protein	-1.71	-1.18	
OEOE_RS02100		Membrane-associated phospholipid phosphatase	1.30	1.41	
OEOE_RS03395		KTSC domain-containing protein			1.26
OEOE_RS03900		ABC transmembrane type-1 domain-containing protein			-1.74
OEOE_RS03915		Predicted hydrolase of the HAD superfamily			1.03
OEOE_RS04145		Predicted acyltransferase	-1.33		-1.55
OEOE_RS04455		ASCH domain-containing protein		-1.12	
OEOE_RS06085		ribonuclease HI			-1.09
OEOE_RS06500		ASCH domain-containing protein			-1.28
OEOE_RS07295		Glycosyltransferase	-1.06		
OEOE_RS08375		Predicted glycosyltransferase			1.06
OEOE_RS08560		Permease of the major facilitator superfamily			-1.08
OEOE_RS08820		Alpha/beta superfamily hydrolase	-1.11		
OEOE_RS08945		Permease of the major facilitator superfamily			-1.07
OEOE_RS09205		RHH_3 domain-containing protein	-1.65	-1.92	
OEOE_RS09255	*ffs*	signal recognition particle sRNA small type		-6.01	4.87

OEOE_RS09285		CHY-type domain-containing protein	-1.29	-1.26	
OEOE_RS09310		MFS transporter	1.84	1.27	
OEOE_RS09345		ABC transporter ATP-binding protein		-1.76	
OEOE_RS09405		ABC transporter domain-containing protein	1.01		
Integral component of membrane- Function unknown					
OEOE_RS00150		Predicted membrane protein		-1.62	-1.09
OEOE_RS00155		Predicted membrane protein		-1.10	
OEOE_RS00255		Predicted membrane protein		-1.37	
OEOE_RS00935		Predicted membrane protein			-2.47
OEOE_RS00965		Predicted membrane protein	-1.10		-2.50
OEOE_RS01545		Predicted membrane protein	1.02		
OEOE_RS01775		Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)		1.30	
OEOE_RS03310		Cell surface protein	2.04		
OEOE_RS03410		Predicted integral membrane protein			-1.12
OEOE_RS07560		bPH_2 domain-containing protein	-1.04		
OEOE_RS08235		Predicted membrane protein	-1.05		
OEOE_RS08800		Predicted multitransmembrane protein			-1.16
OEOE_RS08905		Predicted membrane protein		-1.17	
OEOE_RS08960		Uncharacterized conserved membrane protein		-1.72	
Uncharacterized protein					
OEOE_RS00270	*amaP*	Uncharacterized protein			-1.30
OEOE_RS00335		Uncharacterized protein	-1.12	-1.26	
OEOE_RS00380		Uncharacterized protein		-1.39	
OEOE_RS00385		Uncharacterized protein			-1.26
OEOE_RS00395		Uncharacterized protein			-1.01
OEOE_RS00525		Uncharacterized protein	-1.40	-1.02	-1.16

OEOE_RS00960	Uncharacterized protein	-1.36	-1.60	-1.83
OEOE_RS01090	Hypothetical protein	-1.08		
OEOE_RS01145	Uncharacterized protein			1.61
OEOE_RS01855	Uncharacterized protein	-1.83	-1.89	-1.77
OEOE_RS01880	Uncharacterized protein			-1.23
OEOE_RS01885	Uncharacterized protein			-1.09
OEOE_RS01890	Uncharacterized protein	-1.55		-1.37
OEOE_RS01930	Uncharacterized protein		-1.06	
OEOE_RS02395	Uncharacterized protein			-1.50
OEOE_RS02420	Uncharacterized protein		-1.20	
OEOE_RS02540	Uncharacterized protein			-1.18
OEOE_RS02605	Uncharacterized protein		-1.23	
OEOE_RS03195	Uncharacterized protein		-1.34	
OEOE_RS03305	Uncharacterized protein	-1.68		-1.99
OEOE_RS03405	Uncharacterized protein		-1.35	
OEOE_RS03460	Uncharacterized protein		-1.10	
OEOE_RS03480	Uncharacterized protein	1.25		
OEOE_RS03545	Uncharacterized protein		1.25	
OEOE_RS03890	Uncharacterized protein			-1.80
OEOE_RS03950	Uncharacterized protein			1.03
OEOE_RS03955	Uncharacterized protein			1.19
OEOE_RS04005	Uncharacterized protein	-3.28	-2.33	
OEOE_RS04130	Uncharacterized protein	-1.23		
OEOE_RS04140	Uncharacterized protein			1.10
OEOE_RS04180	Uncharacterized protein	-1.08		
OEOE_RS04575	Uncharacterized protein	-1.12		

OEOE_RS04910		Uncharacterized protein	-1.08		-1.28
OEOE_RS05640		Uncharacterized protein		1.07	-1.13
OEOE_RS05775		Uncharacterized protein	-1.03	-1.03	
OEOE_RS05800		Uncharacterized protein		-1.42	
OEOE_RS06360		Uncharacterized protein		-1.06	
OEOE_RS06385		Uncharacterized protein			-1.65
OEOE_RS06425		Uncharacterized protein		-1.05	
OEOE_RS06705		Uncharacterized protein	-1.26	-1.03	
OEOE_RS07120	*xrtG*	Uncharacterized protein	1.16		
OEOE_RS07305		Hypothetical protein	-1.34		
OEOE_RS08090		Uncharacterized protein	1.06		
OEOE_RS08165		Uncharacterized protein		-1.56	1.20
OEOE_RS08490		Uncharacterized protein			-1.00
OEOE_RS08555		Uncharacterized protein			-1.39
OEOE_RS08570		Uncharacterized protein	-1.02		
OEOE_RS08730		Uncharacterized protein		-1.10	
OEOE_RS08805		Pseudogene	-1.08		-1.75
OEOE_RS08815		hypothetical protein	-1.15		
OEOE_RS08825		Uncharacterized protein	-2.20	-2.19	
OEOE_RS08930		Uncharacterized protein			-1.11
OEOE_RS09115		Uncharacterized protein			1.76
OEOE_RS09200		Hypothetical protein	-1.20	-1.46	
OEOE_RS09300		Uncharacterized protein	-1.23	-1.18	
OEOE_RS09355		Uncharacterized protein	-1.10	-1.45	1.11
OEOE_RS09375		Uncharacterized protein		-1.62	1.30
OEOE_RS09390		Hypothetical protein		1.25	

OEOE_RS09415		Hypothetical protein	-1.18

*locus tag classified in more than a unique COG. Underlined locus tags correspond to matches in transcriptomics and proteomics

Suppl. Table S10. Protein abundance (expressed as fold change) affected during malolactic fermentation by the use of non-*Saccharomyces*, grouped by Clusters of Orthologous Groups (COGs). Sc, Td and Mp correspond to *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, respectively.

Locus tag		Description	log_2FC		
			Sc	Td	Mp
Cellular process and signaling					
Cell cycle. cell division. chromosome partitioning					
OEOE_RS06010		Muramidase (Flagellum-specific)			1.60
OEOE_RS09445	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	-1.57		
		Muramidase with LysM repeats		-2.83	
Cell wall/membrane/envelope biogenesis					
OEOE_RS03220	*ddl*	D-alanine--D-alanine ligase			2.02
OEOE_RS05995		Cell elongation-specific peptidoglycan D.D-transpeptidase		1.53	
OEOE_RS06410		2-dehydro-3-deoxyphosphooctonate aldolase			-1.50
OEOE_RS08160		Small-conductance mechanosensitive channel	1.53		
Defense mechanisms					
OEOE_RS01585*		Dihydrolipoyl dehydrogenase	-2.44	-1.86	
OEOE_RS05130		Beta-lactamase class A			1.66
OEOE_RS06245*		Branched-chain-amino-acid aminotransferase			1.87
OEOE_RS06995*	*rfbC*	dTDP-4-dehydrorhamnose 3.5-epimerase	8.14		
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-3.00	

Intracellular trafficking secretion. and vesicular transport					
-					
Signal transduction mechanisms					
OEOE_RS04290		Aminotransferase	-1.60		
OEOE_RS07990	*dacA*	Diadenylate cyclase			-1.73
OEOE_RS08220		Acetolactate synthase. large subunit	1.82		
Information storage and processing					
DNA replication. recombination and repair					
OEOE_RS00025	*gyrB*	DNA gyrase subunit B			-1.84
OEOE_RS00030	*gyrA*	DNA gyrase subunit A	-1.52		-1.60
OEOE_RS00650		Ribonucleoside-diphosphate reductase			-1.55
OEOE_RS03235	*mutM*	Formamidopyrimidine-DNA glycosylase			-1.56
OEOE_RS04260		Excinuclease ATPase subunit		3.76	
OEOE_RS05365		Ribonucleoside-triphosphate reductase class III catalytic subunit / ribonucleoside-triphosphate reductase	-1.62		-1.88
OEOE_RS06955		DNA helicase			1.53
Post-translational modification. protein turnover and chaperones					
OEOE_RS01385		Heat shock protein Hsp20	4.89	3.32	
OEOE_RS02725		ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	2.03		
OEOE_RS03060		ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones			-1.89
OEOE_RS06305		DnaJ-class molecular chaperone with C-terminal Zn finger domain			1.77
OEOE_RS07610		Protein tyrosine/serine phosphatase			-1.56
OEOE_RS07725		FAD:protein FMN transferase		-2.23	-1.66
OEOE_RS07835		Thiol-disulfide isomerase and thioredoxin			1.61
OEOE_RS07940		Universal stress protein UspA-like nucleotide-binding protein			1.57
Protein biosynthesis. ribosomal structure and biogenesis					

OEOE_RS01655		Acetyltransferase	3.14		
OEOE_RS02120	*serS*	Serine--tRNA ligase			1.51
OEOE_RS03870	*def*	Peptide deformylase	2.05	1.94	2.04
OEOE_RS04345		Predicted GTPase	-2.50		-1.67
OEOE_RS04695		Ribosome-recycling factor	-1.95		
OEOE_RS04750	*era*	GTPase Era		-1.53	
OEOE_RS05975	*efp*	Elongation factor P	-1.83		
OEOE_RS06835		50S ribosomal protein L1	-1.56		
OEOE_RS07520	*cysS*	Cysteine--tRNA ligase			-1.53
OEOE_RS07905		Peptidyl-prolyl cis-trans isomerase	1.94	1.64	1.81
OEOE_RS08485		Cystathionine beta-synthase (Acetylserine-dependent)			-1.82
RNA processing and modification					
OEOE_RS04390		rRNA methylase			1.56
OEOE_RS06200		UPF0173 metal-dependent hydrolase	3.52		1.94
Transcription					
OEOE_RS00285		Transcriptional regulator. HTH and ATP-binding Schlafen-like domain			-1.51
OEOE_RS03800		Transcriptional regulator. xre family	-2.30		
OEOE_RS06440		Transcriptional regulator. GntR family			-1.56
OEOE_RS06735	*rex*	Redox-sensing transcriptional repressor Rex	-1.83	-2.09	-1.59
OEOE_RS07550		Malolactic fermentation system transcription activator	-1.67		-1.63
OEOE_RS07690		Transcriptional regulator. MarR family		1.89	1.87
Metabolism					
Amino acid transport and metabolism					
OEOE_RS01345		Oligoendopeptidase F			-1.51
OEOE_RS01585*		Dihydrolipoyl dehydrogenase	-2.44	-1.86	

OEOE_RS01895		Threonine dehydrogenase-like Zn-dependent dehydrogenase			1.85
OEOE_RS02110		ABC-type antimicrobial peptide transport system. permease component	4.55		
OEOE_RS02115		ABC-type antimicrobial peptide transport system. ATPase component			1.64
OEOE_RS02735		Xaa-Pro aminopeptidase. Metallo peptidase. MEROPS family M24B	2.28	2.02	2.10
OEOE_RS03505		ABC-type antimicrobial peptide transport system. permease component	3.59	4.29	
OEOE_RS03510		ABC-type antimicrobial peptide transport system. ATPase component		5.12	
OEOE_RS03560		Threonine dehydrogenase-like Zn-dependent dehydrogenase		-1.88	
OEOE_RS03725	*glyA*	Serine hydroxymethyltransferase		1.74	
OEOE_RS04030		S-adenosylmethionine synthase	2.18		1.81
OEOE_RS04760		Acetylornithine deacetylase	1.87		
OEOE_RS05245		Oligopeptidase F. Metallo peptidase. MEROPS family M03B			1.69
OEOE_RS05740		Glutathione reductase	1.66		
OEOE_RS06245*		Branched-chain-amino-acid aminotransferase			1.87
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-3.00	
OEOE_RS07535	*luxS*	S-ribosylhomocysteine lyase	4.43	2.14	
OEOE_RS07790		ABC-type dipeptide/oligopeptide/nickel transport system. periplasmic component	3.60	1.83	3.26
OEOE_RS07875		Amino acid ABC transporter substrate-binding protein. PAAT family	1.53		
OEOE_RS08155		Threonine dehydrogenase-like Zn-dependent dehydrogenase	-2.14	-1.54	
OEOE_RS08200	*map*	Methionine aminopeptidase	1.57		
OEOE_RS08240		Threonine dehydrogenase-like Zn-dependent dehydrogenase		-2.52	
OEOE_RS08290		Methionine synthase II (Cobalamin-independent)	-1.63		
OEOE_RS08580		Threonine dehydrogenase-like Zn-dependent dehydrogenase	-1.52	-1.89	-1.70
OEOE_RS08595		Dipeptidase	1.60		
Carbohydrate transport and metabolism					
OEOE_RS00565	*gpmA*	2.3-bisphosphoglycerate-dependent phosphoglycerate mutase	1.69		

OEOE_RS00615		3-hexulose-6-phosphate isomerase	-1.96		
OEOE_RS01055		Cellobiose-specific PTS system IIB component			4.01
OEOE_RS01210		Carbohydrate ABC transporter substrate-binding protein. CUT1 family			-1.62
OEOE_RS01585*		Dihydrolipoyl dehydrogenase	-2.44	-1.86	
OEOE_RS01620		Cellobiose-specific PTS system IIB component	-2.77		
OEOE_RS01985		Phosphoglycerate dehydrogenase-like dehydrogenase	2.82	1.74	
OEOE_RS02230		Phosphotransferase system. mannose/fructose-specific component IIA	-1.54		
OEOE_RS02240		PTS system IID component. Man family		-2.30	
OEOE_RS02510		Zn-dependent alcohol dehydrogenase			-1.64
OEOE_RS03040	*pgi*	Glucose-6-phosphate isomerase			
OEOE_RS03050	*pgk*	Phosphoglycerate kinase	4.66	3.15	
OEOE_RS03325		Acetoin reductase	-1.65		
OEOE_RS04485		Pyruvate oxidase	1.75	1.52	1.64
OEOE_RS05355		Iron-regulated ABC transporter ATPase subunit SufC	-1.54		-1.68
OEOE_RS05695		(R)-2-hydroxyisocaproate dehydrogenase	10.61		6.58
OEOE_RS05700		Phosphoketolase	15.89		10.83
OEOE_RS05700		Phosphoketolase			
OEOE_RS06015	*adhE*	Aldehyde-alcohol dehydrogenase	-1.99	-2.44	-2.19
OEOE_RS06245*		Branched-chain-amino-acid aminotransferase			1.87
OEOE_RS06265		Glycosyltransferase	1.81		
OEOE_RS06270		Glycosyltransferase			1.66
OEOE_RS07015		Glycosyltransferase related enzyme			-2.07
OEOE_RS07030		Carbohydrate ABC transporter ATP-binding protein. CUT1 family		-2.16	-4.15
OEOE_RS07045		Carbohydrate ABC transporter substrate-binding protein. CUT1 family			-1.95
OEOE_RS07050		Glycerophosphoryl diester phosphodiesterase			-3.65
OEOE_RS07130		Glycerate kinase			-1.84

OEOE_RS07250		Glycosyltransferase	-1.64		
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-3.00	
OEOE_RS07730		Acetoin reductase	-2.70	-1.99	-1.74
OEOE_RS07960	*eno*	Enolase	4.67	2.76	
OEOE_RS08225		Alpha-acetolactate decarboxylase	1.74	1.91	
OEOE_RS08245		Branched chain amino acid: 2-keto-4-methylthiobutyrate aminotransferase / fructokinase			2.29
OEOE_RS08575		Sucrose phosphorylase			-4.95
OEOE_RS08585		Alpha-galactosidase	-2.14		-2.05
OEOE_RS08770		Pyruvate oxidase	1.92		
Coenzyme transport and metabolism					
OEOE_RS01335*	*menB*	1.4-dihydroxy-2-naphthoyl-CoA synthase	1.90	1.60	
OEOE_RS06245*		Branched-chain-amino-acid aminotransferase			1.87
Energy production and conversion					
OEOE_RS01580		Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	-1.62		
OEOE_RS01585*		Dihydrolipoyl dehydrogenase	-2.44	-1.86	
OEOE_RS01780		NAD(P) transhydrogenase subunit beta			-1.53
OEOE_RS03185	*atpB*	ATP synthase subunit beta	1.99		
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-3.00	
OEOE_RS07545		Malolactic enzyme	3.01	2.42	2.53
OEOE_RS08870		Cytochrome bd quinol oxidase subunit 1 apoprotein		-1.72	-1.59
Inorganic ion transport and metabolism					
OEOE_RS02640		Phosphate ABC transporter substrate-binding protein. PhoT family		1.84	1.56
OEOE_RS05725		ABC-type metal ion transport system. periplasmic component/surface adhesin			-1.77
OEOE_RS06560		Cation transport ATPase	4.18	3.86	3.66
OEOE_RS06805		Cation transport ATPase		1.52	
OEOE_RS07390		ABC-type Na+ efflux pump. permease component		-2.96	-2.44

OEOE_RS07395		ABC-type uncharacterized transport system. ATPase component			-1.83
OEOE_RS08440		DNA-binding ferritin-like protein (Oxidative damage protectant)			2.23
Lipid transport and metabolism					
OEOE_RS07640	*accD*	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta			2.18
OEOE_RS07645		Biotin carboxylase	1.51	1.63	1.59
OEOE_RS07655		Biotin carboxyl carrier protein of acetyl-CoA carboxylase			3.40
OEOE_RS07660	*fabF*	3-oxoacyl-[acyl-carrier-protein] synthase 2	2.36		2.02
OEOE_RS07670		Malonyl CoA-acyl carrier protein transacylase	1.99		2.44
OEOE_RS07680		Acyl carrier protein			3.21
OEOE_RS07685		3-oxoacyl-[acyl-carrier-protein] synthase 3	2.02	1.55	2.10
Nucleotide transport and metabolism					
OEOE_RS01260	*pyrE*	Orotate phosphoribosyltransferase			-2.08
OEOE_RS01530		dITP/XTP pyrophosphatase	1.58		2.43
OEOE_RS04210		Diadenosine tetraphosphatase-like serine/threonine protein phosphatase			-1.74
OEOE_RS04270		Nucleotide deoxyribosyltransferase			1.80
OEOE_RS05410		Adenylosuccinate lyase			
OEOE_RS06915		Guanylate kinase			1.77
OEOE_RS06990		dTDP-glucose 4.6-dehydratase	1.76		
OEOE_RS07750		Nucleoside ABC transporter ATP-binding protein			-1.71
OEOE_RS07775		Ribokinase	-1.84		
Secondary metabolites biosynthesis. transport. and catabolism					
OEOE_RS01335*	*menB*	1.4-dihydroxy-2-naphthoyl-CoA synthase	1.90	1.60	
OEOE_RS01585*		Dihydrolipoyl dehydrogenase	-2.44	-1.86	
OEOE_RS06995*	*rfbC*	dTDP-4-dehydrorhamnose 3.5-epimerase	8.14		
OEOE_RS07345*	*gnd*	6-phosphogluconate dehydrogenase		-3.00	

Poorly characterized					
General function predicted					
OEOE_RS00015	*yaaA*	S4-like RNA binding protein	-2.66		
OEOE_RS00305		Short-chain alcohol dehydrogenase			-1.64
OEOE_RS00325		Exopolysaccharide biosynthesis protein			-1.61
OEOE_RS00740		Aryl-alcohol dehydrogenase related enzyme	-1.77		-1.83
OEOE_RS01155		Short-chain alcohol dehydrogenase	-1.67	-2.11	
OEOE_RS01790		Short-chain alcohol dehydrogenase	-2.37	-1.69	
OEOE_RS04200		Permease of the major facilitator superfamily		1.82	2.30
OEOE_RS05615		UPF0297 protein OEOE_1166	-1.59		-1.61
OEOE_RS07665		3-oxoacyl-[acyl-carrier-protein] reductase			1.85
OEOE_RS07675		Dioxygenase	1.74		1.77
OEOE_RS07695		3-hydroxymyristoyl/3-hydroxydecanoyl-(Acyl carrier protein) dehydratase		1.90	
OEOE_RS09205		RHH_3 domain-containing protein	-2.65		
Integral component of membrane- Function unknown					
OEOE_RS00855		Predicted membrane protein			1.54
OEOE_RS08235		Predicted membrane protein		-4.03	-3.13
Uncharacterized					
OEOE_RS00260		Uncharacterized protein		-1.95	-2.40
OEOE_RS00310		Uncharacterized protein		-2.07	
OEOE_RS02060		Uncharacterized protein			1.66
OEOE_RS02150		Uncharacterized protein			2.53
OEOE_RS02430		Uncharacterized protein	1.73	1.57	1.64
OEOE_RS03235	*mutM*	Uncharacterized protein			-1.90
OEOE_RS03315		Uncharacterized protein	2.57		

OEOE_RS04195	Uncharacterized protein		2.32
OEOE_RS05710	Uncharacterized protein		
OEOE_RS05710	Uncharacterized protein		
OEOE_RS06555	Uncharacterized protein		3.74
OEOE_RS07165	Uncharacterized protein		-1.72
OEOE_RS07465	Uncharacterized protein		-2.12
OEOE_RS09485	Uncharacterized protein	-2.01	

*locus tag classified in more than a unique COG. Underlined locus tags correspond to matches in transcriptomics and proteomics

CHAPTER V

Nitrogen metabolism of *Oenococcus oeni* in *T. delbrueckii* fermented wines

Aitor Balmaseda[a], Nicolas Rozès[b], Albert Bordons[a],

Hervé Alexandre[c], Cristina Reguant[a,*]

* Corresponding author: cristina.reguant@urv.cat

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[b]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

[c]UMR Procédés Alimentaires et Microbiologiques, Equipe VAlMiS (Vin, Aliment, Microbiologie, Stress), AgroSup Dijon—Université de Bourgogne/Franche-Comté, IUVV, rue Claude Ladrey, BP 27877,21000 Dijon, France

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Abstract

During winemaking process, yeasts consume large quantities of carbon and nitrogen sources throughout alcoholic fermentation (AF), mainly sugars and amino acids. The consumption patterns of these compounds are highly dependent on the fermenting yeasts. Thus, the use of non-conventional yeasts, as *Torulaspora delbrueckii*, could impact in the availability of nutrients after AF. Indeed, after AF, malolactic fermentation (MLF) may eventually occur. The main agent of MLF, *Oenococcus oeni*, has low demand of amino acids and it usually prefers those coming from peptides. We studied the nitrogen metabolism (proteins, peptides, amino acids and biogenic amines (BA)) of three *O. oeni* strains in wines with different inoculation strategies using *T. delbrueckii* in must with different amino acid concentration. We showed an improvement in MLF performance when supplementing amino acids in must. Still, the coinoculation AF strategy was the most inconvenient inoculation for MLF, also causing stuck fermentation. Peptide consumption by *O. oeni* appeared to be dependent on the fermenting medium, related with AF inoculation. Different BA production were observed and 2-phenylethylamine was related also with AF inoculation strategy. Finally, the gene expression of some nitrogen related genes in *O. oeni* was studied and it was upregulated due to the supplementation of amino acids, but it did not enhance the consumption patterns of amino acids in *O. oeni*.

Keywords

Oenococcus oeni, *T. delbrueckii*, amino acids, biogenic amines, peptides, wine

Introduction

Oenococcus oeni is the main species of lactic acid bacteria (LAB) carrying out the malolactic fermentation (MLF) in wine (Davis et al., 1986). This process usually takes place after alcoholic fermentation (AF) undergone by oenological yeasts, transforming grape must into wine (Liu, 2002). During winemaking process, which involves AF and MLF, nutrients from must are used to enable microbial growth (Ribéreau-Gayon et al., 2006). The high fermentative capacity of wine yeasts demands large amount of nutrients to support their growth and metabolic activity. Thus, after AF wine is depleted from the most simple and easy assimilable nutrient sources as sugars, free amino acids, or vitamins (Balmaseda et al., 2018). Besides, AF greatly transforms the physicochemical characteristics of wine by increasing the concentration of some microbial inhibitor compounds as ethanol, sulphur dioxide, medium chain fatty acids or several acids that decrease the pH value (Balmaseda et al., 2018).

Under these restrictive growth conditions, *O. oeni* is able to grow and participate in the winemaking process due to its particular and well adapted metabolic activities (Bech-Terkilsen et al., 2020). It is specialised in the use of alternative energy sources different from sugars. Indeed, the main energy for *O. oeni* in wine is L-malic acid, which is the substrate of MLF (Liu, 2002).

Apart from carbon, nitrogen sources are also necessary for the bacterium to develop. Even if *O. oeni* is considered as a fastidious bacterium due to its large autotrophies, the nitrogen demand of the bacterium is very low (Remize, Augagneur, Guilloux-Benatier, & Guzzo, 2005). Studies on this topic revealed the low consumption of amino acids and the ability of the bacterium to grow without most of them, characteristics that appeared to be strain dependant (Remize et al., 2006). Indeed, wines after MLF usually present higher concentration in free amino acids than after AF (Alcaide-Hidalgo et al., 2008). This is not because an increase in amino acid content by *O. oeni*, but because of the hydrolysis of peptides present in wine/from yeasts and subsequent release of individual amino acids (Manca de Nadra et al., 2005).

Nitrogen composition in wine is mostly composed from proteins, peptides and free amino acids (Ribéreau-Gayon et al., 2006). Protein concentration is low and remains

with no much variation due to a low consumption of wine microorganisms. As introduced before, the amino acid concentration in wine is also very low, around 20 mg N/L. The largest source of nitrogen in wine are peptides (Alcaide-Hidalgo et al., 2008; Martínez-Rodriguez et al., 2001). Peptides can represent up to 100 mg N/L in finished wine and they are the preferent nitrogen source for *O. oeni* in wine.

Another nitrogenous compound found in wine are biogenic amines (BA). BA are polyamines derived from the decarboxylation of the individual amino acids (Landete et al., 2007; Lonvaud-Funel, 1999). Their concentration can represent up to 20 mg/L and they can have a negative effect in the health of the consumer (Restuccia et al., 2018). These compounds can be produced by yeasts or LAB, being more likely related with LAB. From all BA, those more abundant in wine are histamine, cadaverine, 2-phenylethylamine, putrescine and tyramine (Coton et al., 2010; López et al., 2012; Restuccia et al., 2018). Besides, variable but little concentrations of BA, mainly putrescine and cadaverine, can came from grape berries (Bover-Cid et al., 2006; Halász et al., 1994).

As peptides are the main nitrogen source of nitrogen in wine, the understanding of the peptidic metabolism of *O. oeni* is important (Remize et al., 2005). The peptidase activities of *O. oeni* are largely described in literature are seemed to be dependant of the fermenting strain (Manca De Nadra, Farías, Moreno-Arribas, Pueyo, & Polo, 1999; Remize et al., 2006). Besides, they are affected by other exogenous parameters as pH. Those peptides, that are the preferent nitrogen source of *O. oeni* in wine, are the result of the metabolic activities of the previous fermenting yeasts. Thus, the use of different AF inoculation strategies will have an impact in the peptidic fraction, and consequently, in the nitrogen source of the MLF. In this sense, the current trend of using non-*Saccharomyces,* for instance, *Torulaspora delbrueckii*, in winemaking, could have an impact in wine amino acid composition as it has in other wine compounds (Balmaseda et al., 2018). Indeed, *T. delbrueckii* is reported as an interesting starter for red winemaking due to the organoleptic modulation (Benito, 2018a), for instance, polyphenolic composition (**Chapter I: 2**). Besides, the use of this yeast increased the residual free amino acids after AF (Martín-García et al., 2020).

In this study we aimed to evaluate if the use of a particular non-*Saccharomyces*, as *T. delbrueckii* had an impact on the wine amino acid concentration and, thus, impact in *O. oeni*'s nitrogen metabolism. For that purpose, we did fermentations with different AF inoculation regimes in two musts with two amino acid concentrations. We performed MLF with three different *O. oeni* strains and quantify the amino acid concentration before and after MLF. Finally, we studied the relative expression (RE) of some genes related with nitrogen metabolism in *O. oeni* PSU-1.

Materials and methods

Microorganisms

The yeast strains used were *T. delbrueckii* Biodiva (Lallemand, Spain) (TdB), *T. delbrueckii* Viniferm NS-TD (Agrovin S.A., Spain) (TdV) and *S. cerevisiae* Lalvin-QA23 (Lallemand S.L.) (ScQA23). For MLF, *O. oeni* PSU-1 (ATCC BAA-331) (PSU-1), *O. oeni* 217^{T} (CECT217 = ATCC 23279^{T}) (217T) and *O. oeni* Enolab 4783 (4783) were selected. Yeasts were maintained on YPD plates (2% glucose, 2% bacto-peptone, 1% yeast extract, 2% agar, w/v, Panreac Química SLU, Castellar del Vallès, Spain) and the bacteria on MRSmf plates (Martín-García et al., 2020), and all them were stored at 4 °C.

Experimental fermentations

Fermentations were performed with natural concentrated Airén must (Mostos S.A., Tomelloso, Spain) diluted with sterile Milli-Q water until a density of 1,085 ± 1 g/L. Must was supplemented with 0.4 g/L of Nutrient Vit NatureTM (Lallemand, Spain) and pH was adjusted to 3.6. Then, the must was sterilised using 0.1% (v/v) of dimethyl dicarbonate (ChemCruz®, USA) and stored overnight at 4 ºC. The fermenting must amino acid composition was determined by HPLC, as explained below, and a second must was prepared with twice the initial amino acid concentration. The initial must (N1) was supplemented with a solution of the appropriate mixture of amino acids before sterilization to obtain the second must (N2). The concentration in amino acids of each must can be found in Suppl. Table S11.

To undergo the fermentations, different inoculation strategies were employed in the two musts (N1 and N2). First, for sequential inoculation, each *T. delbrueckii* strain was inoculated for a population of 2 x 10^6 CFU/mL and after 48 h of initial inoculation, *S. cerevisiae* QA23 was inoculated for the same population. Second, for coinoculated fermentations, each *T. delbrueckii* strain was inoculated together with *S. cerevisiae* QA23 in must for a population of 2 x 10^6 CFU/mL, respectively. Finally, a control fermentation with *S. cerevisiae* as sole starter was also performed. Fermentations were carried out in triplicate in 1 L flasks containing 950 mL of must, statically at 20 ºC. YPD agar plates were used to calculate the total viable yeast cells, and lysine agar medium (Oxoid LTD., Basingstoke, UK) was used to quantify the non-*Saccharomyces* yeasts, after incubation at 28 ºC for 48 h. AF was considered to have finished when the sugar concentration was below 2 g/L. Then, wines were centrifuged at 10,000 x *g* for 10 minutes. Samples of each replica after AF were frozen at -20 ºC. Then, replicas were blended, filtered (Merck Millipore Steritop™ Sterile Vacuum Bottle-Top Filters, Madrid, Spain) and transferred to 3 sterile flasks. Each flask was inoculated with one of the *O. oeni* strains for a population of 2 x 10^7 CFU/mL. Then, the inoculated wine was divided in six 50 mL tubes and incubated in the same conditions as AFs. Samples were taken every 24 h to monitor the consumption of L-malic acid and the evolution of the bacterial population. Samples were plated on MRSmf and incubated at 27 ºC in a 10% CO_2 atmosphere for 7–15 days. MLF was considered as finished when the L-malic acid was below 0.1 g/L. In addition, three tubes were taken when half of the initial L-malic acid concentration was consumed ($t_{1/2}$) and the other three were taken when MLF was considered as finished (t_f), for gene expression analyses and wine characterization after MLF, respectively.

Cell pellet of *O. oeni* PSU-1 at $t_{1/2}$ was collected. 50 mL of wine were centrifugated at 4,250 x *g* for 20 minutes at 4 ºC. The resulting pellet was washed with 10 mM Tris-HCl pH 8 prepared with diethyl pyrocarbonate-treated water (DEPC), and then frozen in liquid nitrogen and kept at −80 ºC until RNA extraction.

Wine characterization

To monitor AF and MLF, density and L-malic acid were measured with an electronic densimeter (Densito 30PX Portable Density Meter (Mettler Toledo, Spain))

and the multianalyzer Miura One (TDI, SL, Gavà, Spain), respectively. Miura One was also used to determine that AF were finished, quantifying the concentration of glucose + fructose.

Wines were characterized after AF and MLF. pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain). Total and volatile acidity were measured by Fourier Transform Infrared Spectroscopy (FTIR Analysis by FOSS®).

Proteins were quantified using the KDS/BCA assay. Proteins from 500 µL of wine samples after AF and MLF were precipitated, freeze-dried and resuspended as described in Gazzola et al. (2015). Briefly, 5 µL of SDS 10% (w/v) were added to the sample and heated at 100 ºC for 5'. After 125 µL of KCl 1 M were added and the mixture was incubated at room temperature for 2 h. Samples were then centrifugated (12,000 x *g*, 15 minute, 4 ºC) and washed twice with KCl 1 M and once with Milli-Q water. Then, the pellet was freeze-dried and resuspended in 500 µL of Milli-Q water. Proteins were quantified using the BCA-200 Protein Assay kit (Thermo Fisher) following manufacturer's instructions at 562 nm.

Amino acid and peptide analyses

Amino acids were analysed by HPLC as described in Gobert et al. (2017). Briefly, samples were derivatised with AccQTagTM Ultra Derivatization kit, according to the manufacturer's instructions (Waters, USA). Chromatographic separation was performed in a C18 reverse-phase column (AccQ-TagTM Ultra Column, 3.9 × 150 mm) with a fluorometric detector. L-alpha-amino-n-butyric acid (0.1 mM) was used as an internal standard. 2 µL of sample were injected onto the column and the chamber was maintained at 37 ºC.

Wine peptides were quantified as amino acid concentration. 500 µL of HCl 6 M were added to 500 µL of wine sample in a glass vial that was vacuum sealed. Then, the vial was maintained at 110 ºC for 24 h. After, vials were opened, and samples dried with N_2 gas at 37 ºC. The dried pellet was resuspended with 500 µL Milli-Q water and samples were derivatised and analysed as explained above.

For amino acid analyses calculations proline was discarded from the summary of amino acid concentration. Also, Met and Cys were discarded from peptide amino acid

concentrations as they can be degraded during hydrolyses. The summary of Asn and Asp, and Glu and Gln are expressed as Asx and Glx, respectively due to a partial conversion of Asn into Asp and Gln into Glu during the hydrolyses.

Biogenic amines analyses

Cadaverine, histamine, 2-phenylethylamine, putrescine and tyramine were quantified also by HPLC following Gómez-Alonso et al. (2007) with the modifications and conditions described in Bonnin-Jusserand et al. (2012). BA were identified according to the retention times and UV-visible spectral characteristics of the derivatives of the corresponding standards and were quantified by the internal standard method with 2,4,6-trimethylphenethylamine hydrochloride (2 mg/mL).

RNA extraction and RT-qPCR

For RNA extraction, cell pellet of *O. oeni* PSU-1 was defrosted and washed with 10 mM Tris-HCl DEPC water. High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for the extraction following manufacturer's instructions changing the cell lysis for lysozyme dissolved in 10 mM Tris-HCl buffer DEPC, at 50 mg/mL during 30 minutes at 37 ºC (**Chapter III: 2**). Total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Bremen, Germany). Extracted RNA was stored at -80 ºC until RNA analysis.

Some genes related with nitrogen metabolism of *O. oeni*, reported as differentially expressed in previous literature were selected to determine their relative expression (RE) in the different wines (Suppl. Table S12). OligoPerfect Primer Designer (Thermo Fisher) online tool was used for primer design. RT-qPCR was performed according to (**Chapter III: 2**) as described by (Olguín et al., 2009). Four constitutive genes (Margalef-Català et al., 2016a) were evaluated as internal controls. From those, *gyrA* and *gyrB*, which presented the less variation between samples, were selected. The ΔΔCt method (Livak and Schmittgen, 2001) was used to calculate the RE of each gen. We used two reference conditions for determine (i) the impact of *T. delbrueckii*, with the RE in *S. cerevisiae* wine as reference condition, and (ii) the effect of doubling the initial amino acid concentration, with the gene expression in N2 wines referred to N1.

Statistical analysis

All the statistical analyses of the results were performed using the statistics software XLSTAT version 2020.2.3 (Addinsoft, Paris, France). The analysis of variance was carried out by ANOVA with a subsequent Tukey HSD test, to determine the significant differences between the samples: the confidence interval used was 95% and the statistical level of significance was set at $p \leq 0.05$. Next-Generation Clustered Heat Map (NG-CHM) Builder (Ryan et al., 2020) with hierarchical clustering using the Euclidean distance metric with the ward agglomeration method was used for the analyses of amino acid composition, BA production and genes' RE.

Results and Discussion

Fermentations

The duration of AF was extended using *T. delbrueckii* (Figure 34). Control fermentation with *S. cerevisiae* as sole starter took almost the half of the duration of the coinoculation and sequential inoculations (Figure 34, Table 18). In this experiment the supplementation with twice the amino acid concentration (must N2) did not reduce the duration of AF even if an increase in sugar consumption rate was observed (Table 18). This increase was more noticeable in Sc wine and was not observed in TdV wine. In general, the use of *T. delbrueckii* Viniferm resulted in the slowest AF, both in coinoculation and sequential inoculation (Table 18).

The viability of *T. delbrueckii* was longer maintained in sequential inoculations where *S. cerevisiae* was inoculated 48 h after the beginning of the AF (Figure 34). In this sense, no positive effect of doubling the amino acid content was observed in *T. delbrueckii* viability. The AF with *S. cerevisiae* as sole starter presented the highest viable population during the fermentative process (Figure 34). The other inoculation strategies had a lower total viable population similar in coinoculation and sequential inoculation always higher than 10^7 CFU/mL.

Table 18. Alcoholic (AF) and malolactic (MLF) fermentation duration and consumption rate of sugar and L-malic acid, and bacterial viability. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva), TdV (*T. delbrueckii* Viniferm) with C (coinoculation with *S. cerevisiae*) or S (sequentially inoculated with *S. cerevisiae*) refer to the obtained fermented wines in the two must with standard amino acid concentration (N1) and supplemented with twice the initial concentration (N2). PSU-1, 217 and 4783 refer to the MLF strategy where *O. oeni* PSU-1, *O. oeni* 217T, or *O. oeni* Enolab 4783 were inoculated.

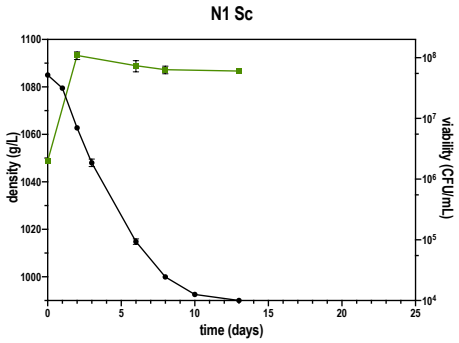
		Fermentation / Total process duration (days)				Consumption rate* (g/L·day)				Viability mid MLF¶ (log CFU/mL)			Viability end MLF (log CFU/mL)		
		AF	PSU-1	217T	4783	AF§	PSU-1	217T	4783	PSU-1	217T	4783	PSU-1	217	4783
N1	Sc	13	-	5 / 18	5 / 18	10.47 ± 0.08^{aB}	-^{fB}	0.36 ± 0.01^{bB}	0.38 ± 0.01^{bB}	6.65 ± 0.01^{eB}	7.45 ± 0.11^{abA}	7.4 ± 0.03^{abcA}	$< 4^{eB}$	7.05 ± 0.01^{cB}	7 ± 0.02^{cB}
	TdBC	23	-	10 / 33	9 / 32	8.02 ± 0.23^{bB}	-^{fA}	0.22 ± 0.01^{dB}	0.31 ± 0.01^{cB}	7.06 ± 0.06^{cdB}	7.54 ± 0.06^{aA}	7.15 ± 0.1^{bcdB}	$< 4^{eA}$	7.5 ± 0.04^{abA}	7.05 ± 0.06^{cB}
	TdVC	23	-	14 / 37	10 / 33	7.77 ± 0.37^{bB}	-^{fA}	0.15 ± 0^{eB}	0.29 ± 0^{cA}	7.38 ± 0.45^{abcA}	7.51 ± 0.01^{abA}	6.89 ± 0.09^{deB}	$< 4^{eA}$	6.65 ± 0.25^{dB}	6.87 ± 0.03^{cB}
	TdBS	20	-	5 / 25	5 / 25	7.71 ± 0.11^{bA}	-^{fB}	0.3 ± 0.01^{cB}	0.3 ± 0^{cB}	7.14 ± 0.01^{bcdB}	7.43 ± 0.04^{abcA}	7.38 ± 0.06^{abcB}	$< 4^{eB}$	7.04 ± 0.06^{cB}	7.32 ± 0.03^{bA}
	TdVS	23	3 / 16	4 / 27	4 / 27	7 ± 0.05^{bA}	0.57 ± 0^{aA}	0.55 ± 0.01^{aA}	0.34 ± 0.04^{bB}	n	n	n	7.47 ± 0.06^{abB}	7.54 ± 0.02^{abA}	7.68 ± 0.05^{aA}
N2	Sc	13	8 / 21	4 / 18	5 / 18	12.54 ± 0.53^{aA}	0.53 ± 0.02^{bA}	0.41 ± 0.03^{deA}	0.66 ± 0.03^{aA}	7.51 ± 0.01^{aA}	7.54 ± 0.05^{aA}	7.52 ± 0.01^{aA}	7.17 ± 0.01^{eA}	7.47 ± 0.01^{bcA}	7.39 ± 0.01^{cA}
	TdBC	23	-	7 / 30	4 / 27	9.13 ± 0.09^{bA}	-^{fA}	0.39 ± 0.03^{deA}	0.36 ± 0.01^{deA}	7.48 ± 0.17^{abA}	7.45 ± 0.04^{abA}	7.48 ± 0.08^{abA}	$< 4^{gA}$	7.53 ± 0.09^{bA}	7.49 ± 0.03^{bA}
	TdVC	23	-	4 / 31	4 / 27	8.61 ± 0.17^{bA}	-^{fA}	0.43 ± 0.02^{cdA}	0.35 ± 0.09^{deA}	7.2 ± 0.1^{dA}	7.32 ± 0.02^{bcdB}	7.46 ± 0.05^{abcA}	$< 4^{gA}$	7.26 ± 0.04^{deA}	7.27 ± 0.04^{dA}
	TdBS	20	4 / 24	6 / 26	6 / 26	7.36 ± 0.41^{cA}	0.43 ± 0.01^{cdeA}	0.35 ± 0^{deA}	0.34 ± 0^{eA}	7.58 ± 0.05^{aA}	7.49 ± 0.04^{abcA}	7.28 ± 0.01^{cdA}	7.5 ± 0.01^{bA}	7.54 ± 0.03^{bA}	7.05 ± 0.01^{fB}
	TdVS	20	4 / 24	6 / 26	4 / 24	6.73 ± 0.15^{cB}	0.5 ± 0.01^{bcB}	0.41 ± 0.03^{deB}	0.43 ± 0^{cdeA}	7.51 ± 0.01^{ab}	7.54 ± 0.05^{a}	7.54 ± 0.05^{a}	7.69 ± 0.01^{aA}	7.56 ± 0.01^{bA}	7.56 ± 0.01^{bB}

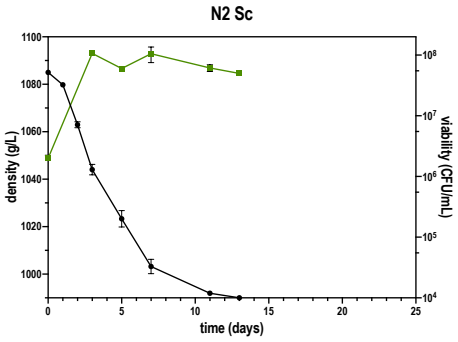
* Calculation based on consumption rate of sugar as density (AF) and L-malic acid (MLF) considering the period of exponential decrease of these values.

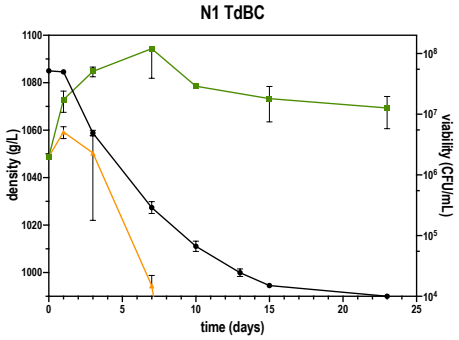
¶ Viability was determined by plating when half of the initial [L-malic acid] was consumed ($t_{1/2}$) and when MLF was considered as finished (t_f) when [L-malic acid] < 0.1 g/L.

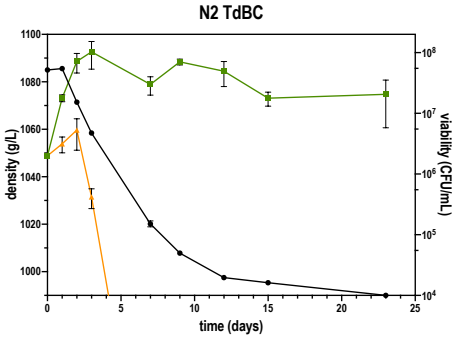
$^{a–g}$ Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences between values of the three strains in the same wine. Uppercase letters correspond to differences among the values of the same *O. oeni* strain in the different wines. § For AF, lowercase letters correspond to differences between values of the different AF strategies in the same must. Uppercase letters correspond to differences among the values of the same AF strategy in the different wines.

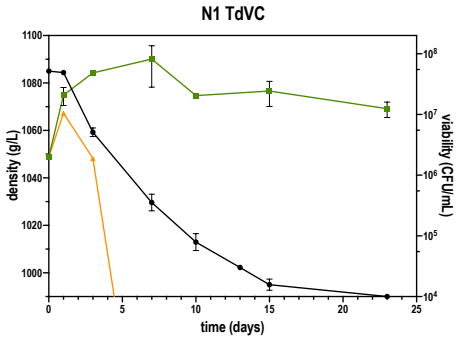
-: no finished MLF. n: no data.

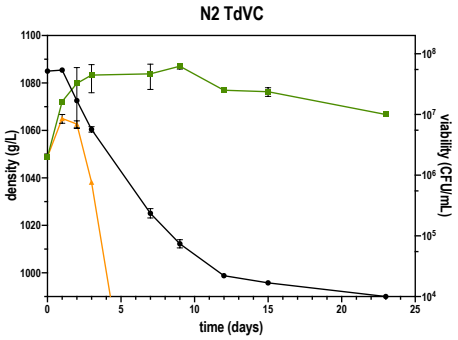

N1 Sc
density (g/L)
viability (CFU/mL)
time (days)

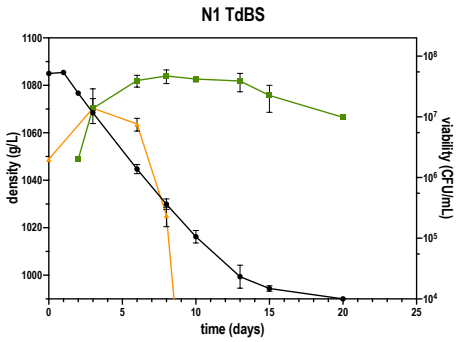

N2 Sc
density (g/L)
viability (CFU/mL)
time (days)

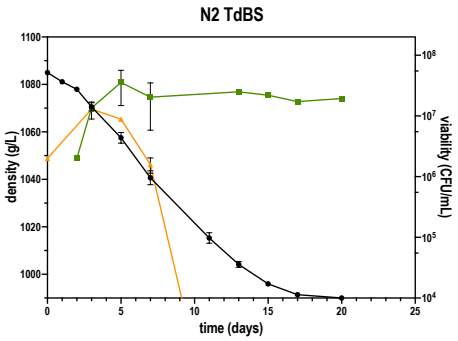

N1 TdBC
density (g/L)
viability (CFU/mL)
time (days)

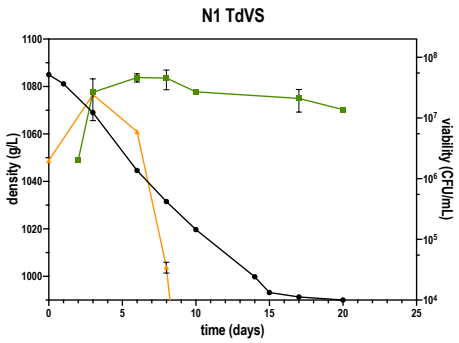

N2 TdBC
density (g/L)
viability (CFU/mL)
time (days)


N1 TdVC
density (g/L)
viability (CFU/mL)
time (days)


N2 TdVC
density (g/L)
viability (CFU/mL)
time (days)


N1 TdBS
density (g/L)
viability (CFU/mL)
time (days)


N2 TdBS
density (g/L)
viability (CFU/mL)
time (days)


N1 TdVS
density (g/L)
viability (CFU/mL)
time (days)

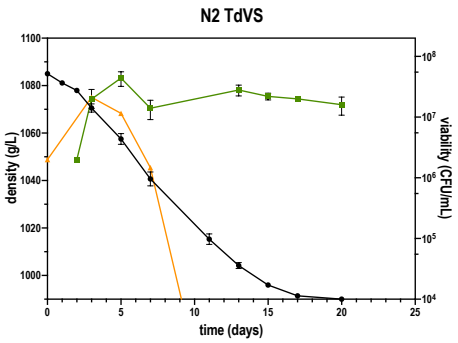

N2 TdVS
density (g/L)
viability (CFU/mL)
time (days)

Figure 34. Alcoholic fermentation dynamics where density decrease (black) and yeast viability are represented for the two used species: *S. cerevisiae* (green) and *T. delbrueckii* (orange) in the must with standard amino acid concentration (N1) and must supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates ± SD. Sc, TdB and TdV refers to *S. cerevisiae* QA23, *T. delbrueckii* Biodiva- *S. cerevisiae* QA23 and *T. delbrueckii* Viniferm- *S. cerevisiae* QA23, respectively. C and S after TdB/TdV means coinoculation of the two yeasts and sequential inoculation, respectively.

The obtained wines were then inoculated with three different *O. oeni* strains to undergo MLF. Larger differences in MLF and a positive effect of doubling the initial amino acid concentration were observed (Figure 35, Table 18). MLF in N1 wines showed a general delay when it was performed in the coinoculated wines. Besides, PSU-1 strain caused stuck fermentations in all conditions except in TdV wine. The best condition for the other two strains was also TdV wine. This wine showed the lowest duration of MLF for 217T and 4783 strains (Figure 35, Table 18).

In general, N2 reduced the duration of the MLF process (Figure 35, Table 18). According to PSU-1 strain, all fermentations finished except for TdBC and TdVC wines, which also were stuck. Sequential inoculation (TdBS, TdVS) for PSU-1 was the most convenient AF strategy since it finished in half the time of that observed in Sc wine. Moreover, the delays in coinoculated wines observed in N1 disappeared in N2 with the other two strains. The durations were reduced as the differences did.

Viability of the three *O. oeni* strains was maintained around 10^7 CFU/mL during the fermentative process except for those stuck MLFs that lost the viability and were not able to finish the fermentation (Table 18). Higher population density was observed in those wines coming from N2. Besides, it is also interesting to point that the TdVC wines from N1 showed a population lower than 10^7 CFU/mL with 217T and 4783 strains, still enough for conclude MLF.

General oenological parameters did not show large differences in the tested conditions (Suppl. Table S13). L-malic acid of N1 wines was significantly lower in Td wines, both in coinoculated and sequential AF, than in Sc wines. In N2 wines, the decrease of L-malic acid at the end of AF was observed only in Td wines with sequential inoculation. Also, a significant increase in ethanol content was observed in coinoculation with *T. delbrueckii* in N2. *T. delbrueckii* is usually reported as a yeast, which can reduce the alcoholic content of wines (Benito, 2018a). Nevertheless, the

fermenting medium and the inoculation strategy is crucial to achieve that reduction (Martín-García et al., 2020).

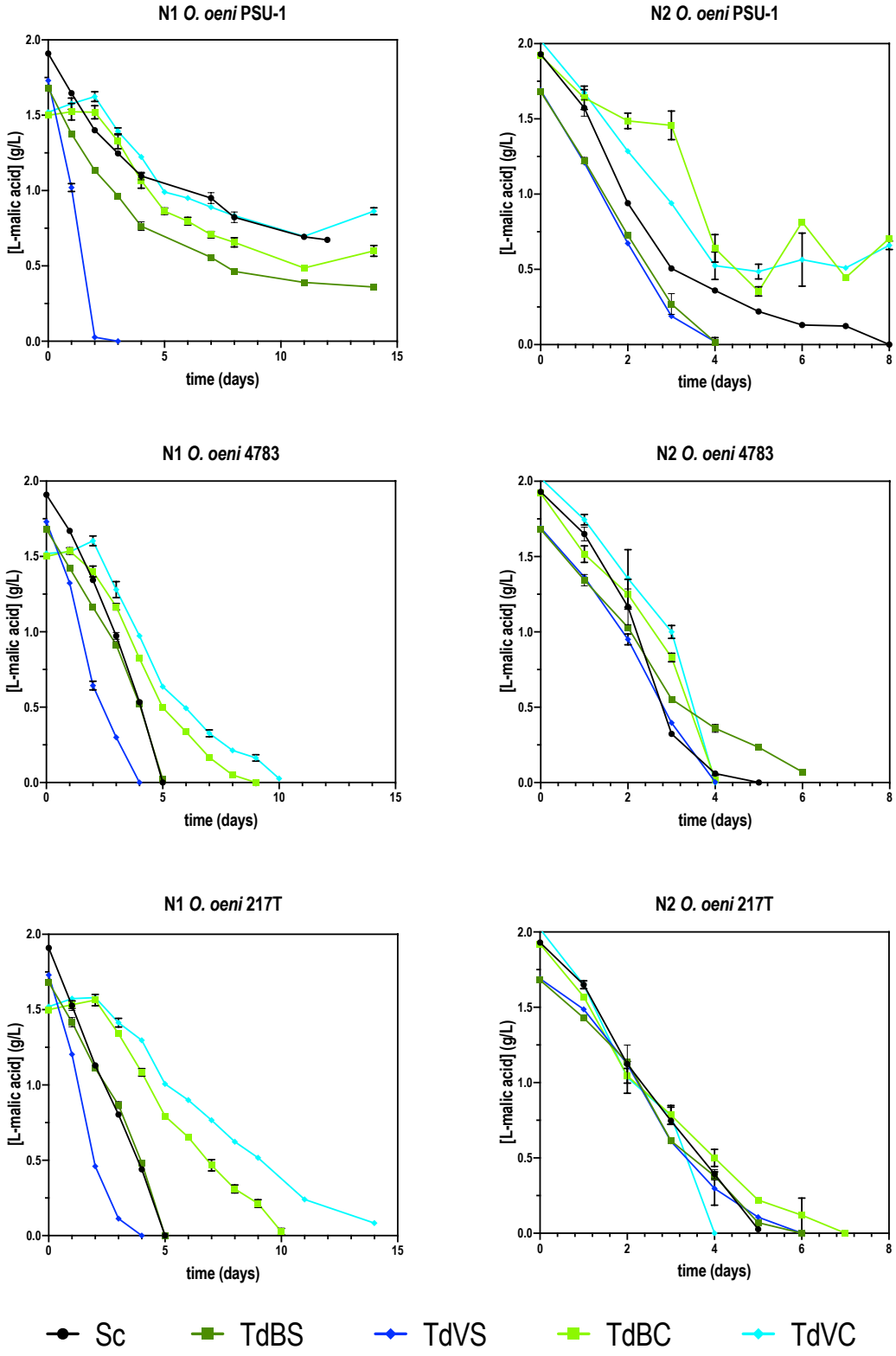


Figure 35. Malolactic fermentation dynamics where L-malic consumptions of *O. oeni* PSU-1, 217T and 4783 in the must with standard amino acid concentration (N1) and must supplemented with twice the initial concentration (N2) are represented. Values shown are the mean of triplicates ± SD. Abbreviations are the same as in Figure 34.

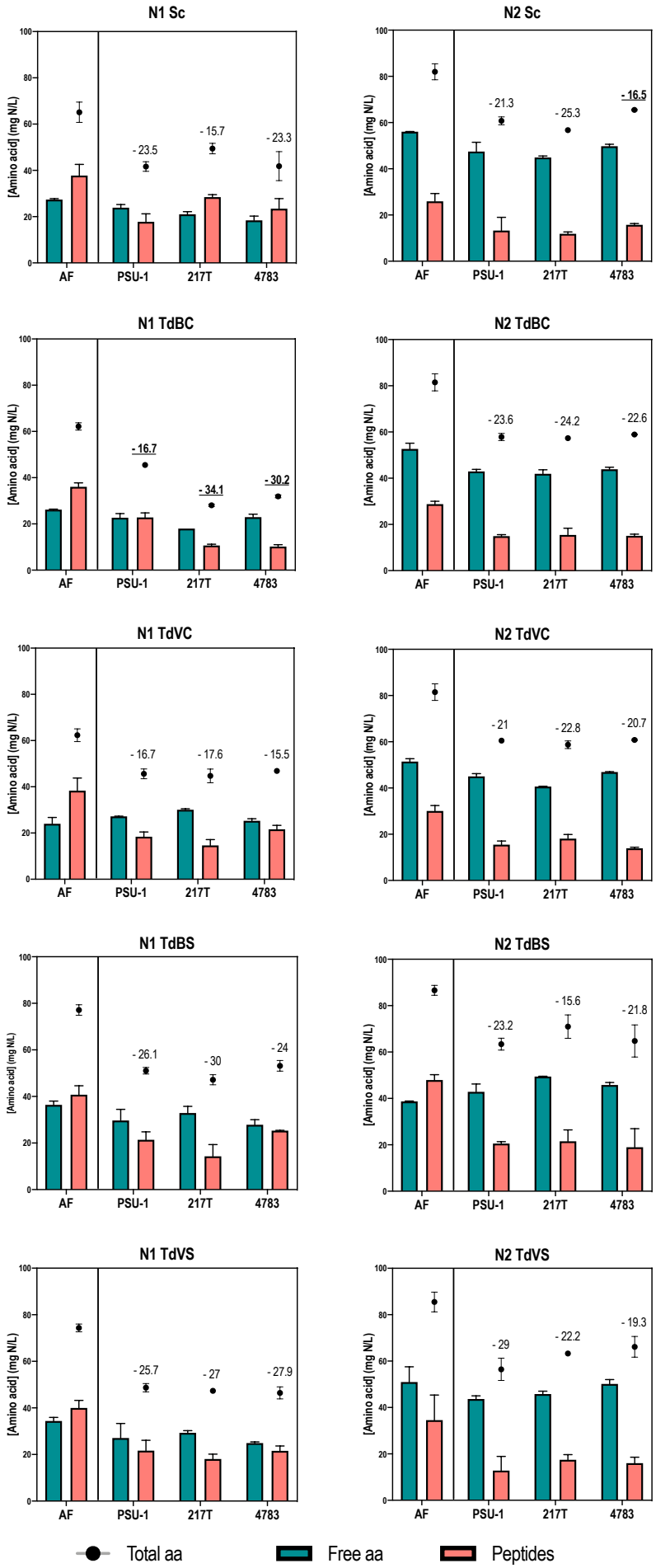

N1 Sc
N2 Sc
N1 TdBC
N2 TdBC
N1 TdVC
N2 TdVC
N1 TdBS
N2 TdBS
N1 TdVS
N2 TdVS
[Amino acid] (mg N/L)
AF
PSU-1
217T
4783
- 23.5
- 15.7
- 23.3
- 21.3
- 25.3
- 16.5
- 16.7
- 34.1
- 30.2
- 23.6
- 24.2
- 22.6
- 16.7
- 17.6
- 15.5
- 21
- 22.8
- 20.7
- 26.1
- 30
- 24
- 23.2
- 15.6
- 21.8
- 25.7
- 27
- 27.9
- 29
- 22.2
- 19.3
Total aa
Free aa
Peptides

Figure 36. Total amino acid concentration (mg N/L) distribution of wines before (AF) and after MLF with *O. oeni* strains (PSU-1, 217T and 4783) in the must with standard amino acid concentration (N1) and must supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates ± SD. The number above total amino acid concentration (black dot) after MLF (PSU-1, 217T and 4783) refers to consumed total amino acid concentration during MLF. Underlined and bolded values are significantly different between wines after MLF ($p \leq 0.05$ according to a Tukey HSD post-hoc comparison). Other abbreviations are the same as in Figure 34.

Free and peptide amino acid concentrations

The different preferences and demands of nitrogen by the fermenting yeasts will determine the amino acid composition found in wine. Moreover, the use of *T. delbrueckii* has been previously linked with higher free amino acid availability in wine (Martín-García et al., 2020). In this study the different inoculation strategies produced wines with different amino acid composition in both, free and peptide amino acids (Figure 36). In wines coming from N1 the concentration of free amino acids was in average of 20 mg N/L whereas the peptidic fraction showed higher nitrogen reservoir, representing an average of around 40 mg N/L. In contrast, the remaining free amino acids after AF in N2 was higher than quantified in N1 (Figure 36). In these wines, the peptidic fraction was similar to N1 wines except for TdBS where the peptide amino acids were higher than the free fraction and higher than observed in TdBS from N1. In general, a little decrease in the peptide amino acid concentration was observed when comparing with N1 wines after AF. In this sense, we showed that the addition of free amino acids in must had an increase in total amino acid composition by only increasing the free amino acid concentration (Figure 36). Besides, in N1 the only wine significantly different from the control condition (Sc wine) was TdBS in concordance with Martín-García et al. (2020).

In this study we observed that amino acid consumption during AF by yeasts did not exhaust all the amino acid concentration, thus increasing their availability for the subsequent MLF. It is well known that amino acids are essential for yeasts during AF (Ribéreau-Gayon et al., 2006). They are largely consumed, and high amino acid demands are related with high fermentative capacity (Roca-Mesa et al., 2020). Nevertheless, peptide composition in wine by different yeasts has not already been reported. In this study, the use of *T. delbrueckii* modified the amino acid concentration from peptides (Suppl. Figure S9). In N1 wines, increased the concentration of Glx, Arg,

Asx and His with respect to *S. cerevisiae* wine after AF. Nevertheless, the composition was different in N2 wines where the composition of TdBS and TdVC were very different from the others due to a general increased concentration, mainly due to Glx and Ala (Suppl. Figure S9).

Total amino acid concentration decreased after MLF (Figure 36). During this process, *O. oeni* consumes amino acids. It is interesting to note that *O. oeni* consumed the amino acids coming from peptides and the free amino acid concentration remained similar (Figure 36, Suppl. Figure S9). *O. oeni* has a vast set of peptidases that releases free amino acids to the media during MLF (Margalef-Català et al., 2016a). Indeed, the bacterium can grow with peptides as sole nitrogen source, proving the importance of this nitrogen compounds in the metabolism of *O. oeni* (Martínez-Rodríguez et al., 2001; Remize et al., 2006). The amino acid consumption patterns were mainly affected by the fermenting medium. The three tested strains consumed similar total amino acid concentration. We only observed a different consumption pattern in 4783 strain in N2 Sc wine, and in N1 TdBC wine all strains behaved differently (Figure 36). Besides, the total concentration of each amino acid was similar in TdBC, TdVC, and Sc, different from TdBS and TdVS (Suppl. Figure S9). In stuck MLFs there was also a consumption in total amino acid concentration (Figure 36), which can be related with the first fermentative stages where *O. oeni* was still viable and metabolically active (Figure 35). In this sense, Sc wine and coinoculated wines from N1 presented higher demands of amino acids than sequential inoculated ones. Nevertheless, the consumption of PSU-1 was similar to the other two strains in the same wines, thus, only responding to the particular medium.

The consumption of amino acid during MLF was mainly due to a reduction in peptide concentration (Figure 36). Nevertheless, even if the total consumption of amino acids was similar in all wines, the amino acid composition of peptides in each wine was different, as it was also the consumption of *O. oeni*. Figure 37 shows the comparison of the amino acid consumption profiles during MLF in the different wines by the three different *O. oeni* strains. Overall, a consumption of every amino acid was observed. Moreover, a general higher consumption was observed in N2 wines (Figure 37B) regarding to N1 wines (Figure 37A). This is interesting to point since there was a higher concentration of available of free amino acids in N2 regarding to N1 wines

(Figure 36). Probably, the increased concentration of amino acids found in wine leaded to an enhanced amino acid metabolism in *O. oeni*, which increased the hydrolysis of peptides.

The consumption patterns from peptide amino acids in N1 wines were clearly dependent on the yeast inoculation strategy. A high consumption of Arg, Glx and His, together with a low general consumption of the rest of the amino acids, grouped the wines sequentially inoculated with *T. delbrueckii* and *S. cerevisiae* (Cluster I, Figure 37A). Meanwhile, the wines inoculated only with Sc and all the wines coinoculated with the two yeast species clustered apart (Cluster II, Figure 37A).

The consumption patterns in N2 wines were more heterogeneous. Although some similarities according to the yeast inoculation strategy were observed, there was not such a clear relationship as in N1 wines (Figure 37B). All wines were clustered together in Cluster II except for TdBS wines, grouped in Cluster I. Interestingly, TdBS presented the highest peptide concentration from all conditions (Figure 36), which also presented the highest consumption of amino acids (Figure 37B). In N2 wines, the most consumed amino acids were Glx, in mainly all conditions, and Arg and Thr in TdBS wines.

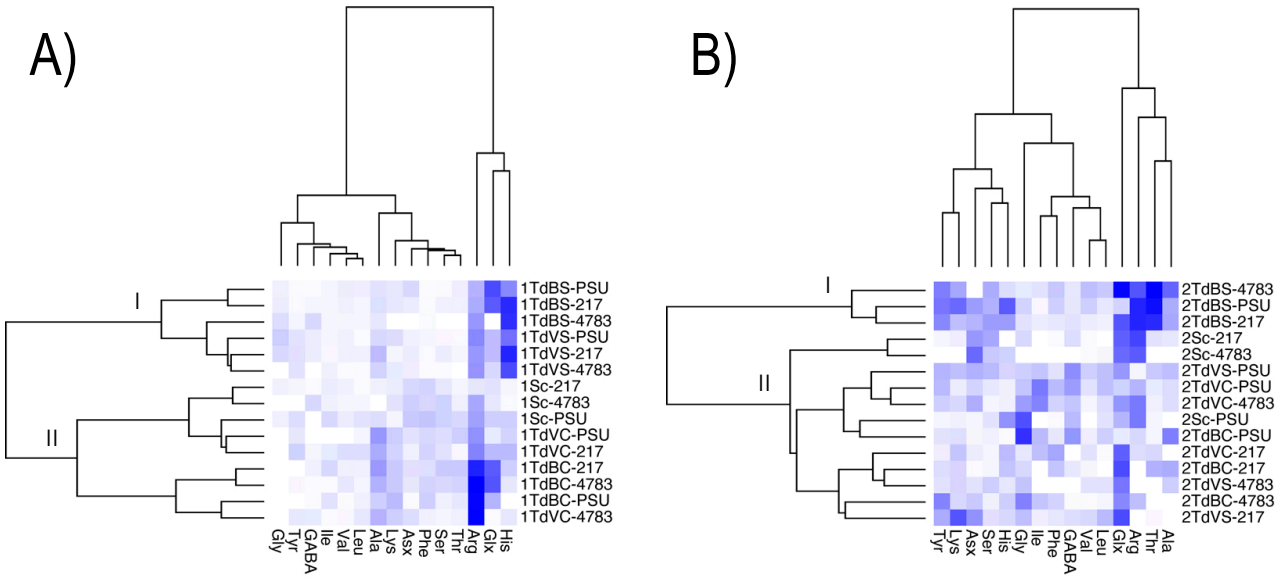


Figure 37. Heat map and clusterization of amino acid consumption (mg N/L) of wine peptides. A) Consumption in N1 must. B) Consumption in N2 must. Increasing colour intensity means higher amino acid consumption. Dendrogram total length correspond to 100% of similarity. Other abbreviations are the same as in Figure 34.

Altogether, the preference for peptides by *O. oeni* under oenological conditions was confirmed. In this study we could observe the consumption patterns of *O. oeni* in wines fermented with different AF strategies. Nitrogen metabolism is one of the main metabolisms affected by wine like conditions, and also reported to be affected by the use of non-*Saccharomyces*. We observed a complex, which was also influenced by the initial amino acid concentration. Indeed, high amino acid concentrations were also responsible for an increased peptide consumption. Further research is needed to better understand this complex metabolism under oenological conditions.

Biogenic amines

From the five BA studied, cadaverine, 2-phenylethylamine, putrescine and tyramine, were detected, but not histamine (Table 19). It is worth nothing that no BA content was detected in must, except from 3-4 mg/L of putrescine (data not shown). That is why the amount of putrescine detected after AF is not consequence of the inoculated fermenting yeasts. Overall, the production of BA was not related with a higher availability of its amino acid precursor or to its increased consumption.

The production of BAs was low in this work except for putrescine (Table 19). Only wines fermented with *O. oeni* 4783 showed an increased putrescine content. This strain is characterized by the presence and expression of the *odc* gene (Franquès et al., 2018). Indeed, the strain 4783 is the only tested strain owning this gene of the study. It is interesting to point that some ornithine decarboxylases can use, apart from ornithine, lysine as substrate, and consequently produce cadaverine (Romano et al., 2012). In this sense, it was also observed a significant increase in cadaverine in wines after MLF fermented with *O. oeni* 4783. Thus, the increased cadaverine content in 4783 fermented wines should be related with a non-specific substrate ODC enzyme. The high production of putrescine of 4783 wines clustered all those wines in the same group (Table 19). The production of putrescine only depended on the fermenting *O. oeni* strain (presence or absence of *odc*), regardless the AF inoculation or the initial amino acid concentration. Little amounts of cadaverine were also detected after AF in concentrations of 0.11-0.21 mg/L in all wines after AF which was maintained after MLF in *O. oeni* PSU-1 and 217T fermented wines (Table 19). Indeed, cadaverine can be detected after AF and usually not increase after MLF (López et al., 2012).

Table 19. Biogenic amine (BA) concentration (mg/L) in obtained wines the two must with standard amino acid concentration (N1) and supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates ± SD. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva), TdV (*T. delbrueckii* Viniferm) with C (coinoculation with *S. cerevisiae*) or S (sequentially inoculated with *S. cerevisiae*) refer to the obtained fermented wines. P, 217T and 4783 refer to the MLF strategy where *O. oeni* PSU-1, *O. oeni* 217T, or *O. oeni* Enolab 4783 were inoculated.

	Cadaverine		Phenylethylamine		Putrescine		Tyramine		Total BA	
	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2
Sc	0.13 ± 0.02^{aA}	0.11 ± 0.01^{aA}	0.75 ± 0.05^{bcdB}	0.53 ± 0.02^{defA}	3.52 ± 0.42^{aA}	4.53 ± 0.34^{aA}	n.d.^{aA}	1.17 ± 0.54^{bB}	4.4 ± 0.48^{a}A	6.33 ± 0.18^{cdeB}
Sc-P*	0.17 ± 0.04^{aA}	0.1 ± 0.01^{aA}	0.77 ± 0.02^{dB}	0.51 ± 0.01^{cdefA}	4.11 ± 0.02^{aA}	4.34 ± 0.15^{aA}	n.d.^{aA}	0.72 ± 0.01^{bB}	5.1 ± 0.04^{abA}	5.68 ± 0.14^{abcdB}
Sc-217T	0.14 ± 0.01^{aA}	0.12 ± 0.01^{aA}	0.74 ± 0.08^{bcdA}	0.58 ± 0.01^{fA}	4.46 ± 0.55^{aA}	4.64 ± 0.21^{aA}	n.d.^{aA}	1.16 ± 0.15^{bB}	5.34 ± 0.64^{abcA}	6.51 ± 0.06^{deA}
Sc-4783	0.51 ± 0.08^{cA}	0.47 ± 0.02^{fA}	0.79 ± 0.08^{dB}	0.52 ± 0.02^{cdefA}	11.27 ± 0.95^{bA}	11.67 ± 0.65^{cA}	n.d.^{aA}	0.95 ± 0.05^{bB}	12.56 ± 1.11^{dA}	14.08 ± 1.29^{hA}
TdBC	0.16 ± 0.07^{aA}	0.13 ± 0.01^{abA}	0.38 ± 0.17^{abA}	0.39 ± 0.01^{abcdeA}	4.76 ± 0.75^{aA}	4.97 ± 0.16^{aA}	1.51 ± 0.36^{deB}	n.d.^{aA}	6.82 ± 1.35^{bcA}	5.49 ± 0.17^{abcdA}
TdBC-P*	0.12 ± 0.01^{aA}	0.11 ± 0.01^{aA}	0.28 ± 0.01^{aA}	0.25 ± 0.02^{aA}	5.05 ± 0.3^{aA}	4.12 ± 0.16^{aA}	1.83 ± 0.13^{eB}	n.d.^{aA}	7.28 ± 0.45^{cB}	4.48 ± 0.19^{a}A
TdBC-217T	0.13 ± 0.01^{aB}	0.1 ± 0.01^{aA}	0.29 ± 0.01^{aA}	0.43 ± 0.2^{bcdefB}	5.06 ± 0.1^{aA}	4.74 ± 0.04^{aA}	1.68 ± 0.43^{deB}	n.d.^{aA}	7.15 ± 0.33^{bcB}	5.99 ± 0.12^{bcdA}
TdBC-4783	0.39 ± 0.03^{bcA}	0.24 ± 0.04^{cA}	0.4 ± 0.02^{abcA}	0.38 ± 0.15^{abcdeA}	11.39 ± 0.61^{bA}	11.35 ± 0.56^{cA}	1.21 ± 0.05^{cdB}	n.d.^{aA}	13.4 ± 0.71^{dA}	11.97 ± 0.67^{gA}
TdVC	0.13 ± 0.01^{aA}	0.14 ± 0.01^{abA}	0.4 ± 0.01^{abcA}	0.37 ± 0.01^{abcdA}	4.68 ± 0.03^{aA}	4.79 ± 0.04^{aA}	n.d.^{aA}	1.97 ± 0.05^{bB}	5.2 ± 0.01^{abcA}	7.26 ± 0.01^{eB}
TdVC-P*	0.11 ± 0.01^{aA}	0.12 ± 0.02^{aA}	0.39 ± 0.01^{abA}	0.32 ± 0.06^{abA}	4.53 ± 0.18^{aA}	4.72 ± 0.17^{aA}	n.d.^{aA}	n.d.^{aA}	5.03 ± 0.18^{abA}	5.16 ± 0.21^{abcA}
TdVC-217T	0.12 ± 0.02^{bA}	0.13 ± 0.01^{abA}	0.28 ± 0.01^{a}A	0.4 ± 0.05^{abcdeA}	4.83 ± 0.05^{aA}	4.84 ± 0.01^{aA}	n.d.^{aA}	n.d.^{aA}	5.23 ± 0.08^{abcA}	5.37 ± 0.05^{abcdA}
TdVC-4783	0.37 ± 0.01^{bcA}	0.32 ± 0.02^{deA}	0.28 ± 0.01^{a}A	0.36 ± 0.01^{abcB}	11.22 ± 0.3^{bA}	11.29 ± 0.45^{cA}	1.38 ± 0.01^{deB}	n.d.^{aA}	13.25 ± 0.32^{dA}	11.97 ± 0.44^{gA}
TdBS	0.17 ± 0.01^{aA}	0.18 ± 0.01^{bA}	0.88 ± 0.06^{dB}	0.52 ± 0.01^{cdefA}	4.47 ± 0.53^{aA}	4.33 ± 0.27^{aA}	0.63 ± 0.15^{bcB}	n.d.^{aA}	5.9 ± 1.12^{abcB}	5.03 ± 0.27^{abA}
TdBS-P*	0.21 ± 0.01^{aB}	0.1 ± 0.01^{aA}	0.9 ± 0.02^{dB}	0.3 ± 0.01^{abA}	4.67 ± 0.13^{aA}	4.28 ± 0.05^{aA}	n.d.^{aA}	n.d.^{aA}	6.27 ± 0.11^{abcB}	4.69 ± 0.1^{a}A
TdBS-217T	0.21 ± 0.02^{aB}	0.12 ± 0.01^{aA}	0.94 ± 0.01^{dB}	0.53 ± 0.04^{efA}	4.99 ± 0.22^{aA}	4.58 ± 0.05^{aA}	n.d.^{aA}	n.d.^{aA}	6.13 ± 0.25^{abcB}	5.24 ± 0.01^{abcA}
TdBS-4783	0.7 ± 0.04^{dB}	0.32 ± 0.01^{dA}	0.88 ± 0.01^{dB}	0.41 ± 0.01^{abcdeA}	12.11 ± 0.48^{bB}	9.63 ± 0.33^{bA}	0.54 ± 0.13^{abB}	n.d.^{aA}	14.01 ± 0.08^{dB}	10.36 ± 0.31^{fA}
TdVS	0.13 ± 0.04^{aA}	0.11 ± 0.01^{aA}	0.63 ± 0.26^{abcdA}	0.45 ± 0.04^{abcdefA}	4.64 ± 0.01^{aA}	4.53 ± 0.11^{aA}	1.48 ± 0.21^{deB}	n.d.^{aA}	6.92 ± 0.52^{bcB}	5.09 ± 0.15^{abA}
TdVS-P	0.16 ± 0.01^{aB}	0.11 ± 0.01^{aA}	0.78 ± 0.01^{dB}	0.43 ± 0.01 ^{abcdefA}	4.77 ± 0.02^{aB}	4.45 ± 0.01^{aA}	1.49 ± 0.03^{deB}	n.d.^{aA}	7.21 ± 0.02^{bcB}	4.97 ± 0.01^{abA}
TdVS-217T	0.21 ± 0.01^{aB}	0.12 ± 0.01^{aA}	0.77 ± 0.03^{cdB}	0.42 ± 0.01 ^{abcdefA}	4.63 ± 0.01^{aA}	4.25 ± 0.07^{aA}	1.44 ± 0.03^{deB}	n.d.^{aA}	7.06 ± 0.08^{bcB}	5 ± 0.34^{abA}
TdVS-4783	0.69 ± 0.01^{dB}	0.38 ± 0.02^{eA}	0.59 ± 0.22^{abcdA}	0.44 ± 0.02 ^{abcdefA}	11.6 ± 0.44^{bA}	10.74 ± 0.46^{bcA}	1.51 ± 0.14^{deB}	n.d.^{aA}	14.39 ± 0.37^{dB}	11.21 ± 0.01^{fgA}

* wines with stuck MLF at least in one of the musts.

[a–g] Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences between values of wines from the same must. Uppercase letters correspond to differences among the values of the inoculation condition from the two musts. Values in bold are significantly different from the value at the end of AF in the same wine (before MLF).

n.d.: not detected

2-phenylethylamine increased after AF and no changes were observed as consequence of MLF (Table 19). 2-phenylethylamine appears after AF and usually remains without changes after MLF (López et al., 2012). Interestingly, its concentration was increased in N1 wines and was dependent of the inoculation strategy. Lower concentrations were detected in wines sequentially inoculated with *T. delbrueckii* in contrast to coinoculated and control wines (Table 19). Tyramine content exhibited a heterogeneous behaviour, it was detected in some wines after AF or MLF, and no correlation could be found with the detected concentration (Table 19).

Relative expression (RE) of nitrogen related genes in *O. oeni* PSU-1

To better understand the nitrogen metabolism of *O. oeni* under oenological conditions, we selected some genes to study their RE (Suppl. Table S12). From the three strains used, we selected *O. oeni* PSU-1 as representative strain as the three of them had a similar consumption amino acid consumption pattern and due to its different MLF performances. The selection of genes was based on previous works where the genes classified in the Clusters of Orthologous Groups (COG) for amino acid transport and metabolism exhibited differential expression in terms of mRNA or protein abundance (**Chapter IV**; Margalef-Català et al., 2016a; Olguín et al., 2015). These genes encode for two peptidases (OEOE_RS08595 and OEOE_RS2735), a peptide transporter (OEOE_RS02110), an amino acid/polyamine/organocation transporter (OEOE_RS05625) and for the glutamine synthase (OEOE_RS04565).

We studied their RE considering two possible effects: (i) *T. delbrueckii* impact, and (ii) amino acid supplementation (Figure 38). In all conditions, *O. oeni* PSU-1 exhibited an upregulation of the studied genes, which significant upregulations (RE > 2) are coloured in Figure 38.

Considering the effect of *T. delbrueckii*, *O. oeni* exhibited a very increased transcriptional response in TdBS wine of Must 1, which was also clustered very far from the other conditions (Figure 38A). TdBC wines from N1 and N2 were clustered together and had very low response. The other conditions had a significant upregulation and were clustered together. The hierarchical clustering of the RE of each gene revealed a similar response pattern in the two transporters.

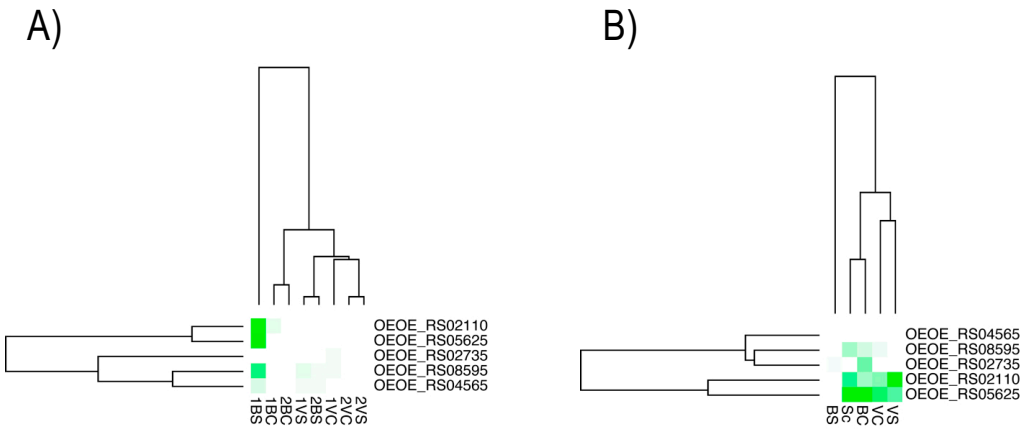


Figure 38. Heat map and clusterization of the relative expression of some nitrogen related genes in *O. oeni* PSU-1. A) Effect of *T. delbrueckii*: RE is calculated with *S. cerevisiae* as control condition, where the gene expression of *O. oeni* PSU-1 in mixed fermentation is referred to *S. cerevisiae* in Must 1 (1) and *S. cerevisiae* in Must 2 (2). B) Effect of amino acid supplementation: RE is calculated with the gene expression of *O. oeni* PSU-1 in each wine of N2 referred to the gene expression in each wine in N1. Dendrogram total length correspond to 100% of similarity. Other abbreviations are the same as in Figure 34.

When comparing the RE of each wine in N2 compared to N1 wines, we observed a general upregulation of all genes in all conditions (Figure 38B). Indeed, an increased amino acid consumption was observed in N2, mainly by the consumption of peptides (Figure 4). The exception was TdBS, which did not present any differentially expressed gene. This is related to a very high expression of each gene in N1 wine. In this case, the RE of those genes in the studied conditions clustered Sc together with TdBS, and TdVC and TdVS, reporting a similar response to amino acid supplementation in Sc and TdB wines, which was more different in TdV wines. In this comparison, the expression patterns of the genes were clustered based on their function: the two peptidases together, the two transporters together, and glutamine synthase (Figure 38B).

The general upregulation observed in this work could indicate that the studied mechanisms (peptidases, peptide and amino acid transporters and glutamine

synthase) are a general stress response to wine in *O. oeni* as described in the previous works (**Chapter IV**; Margalef-Català et al., 2016a; Olguín et al., 2015). Thus, it demonstrates that the peptide composition and utilization is a key factor in *O. oeni* survival.

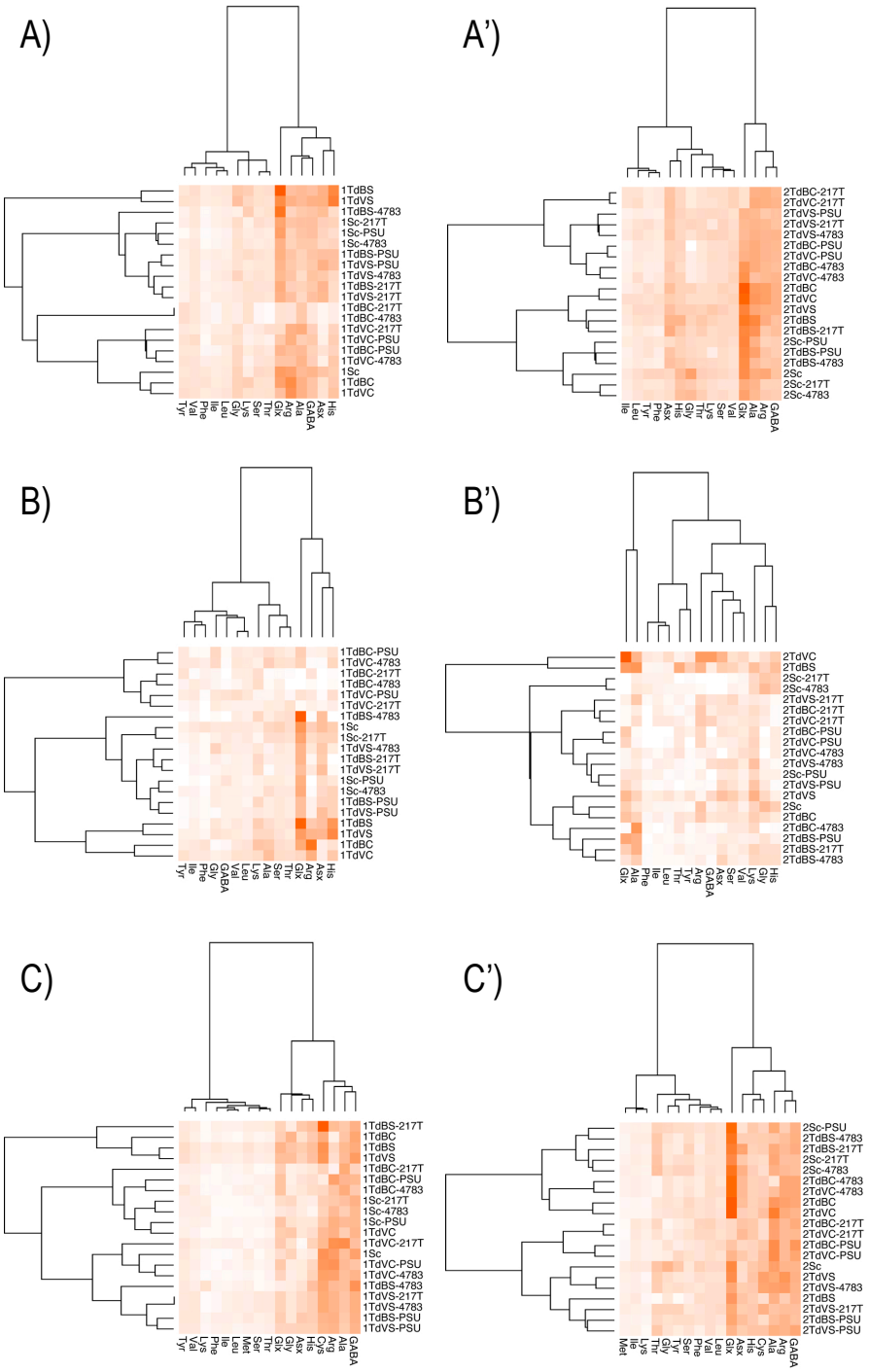
Conclusion

Nitrogen metabolism in *O. oeni* has been a recurrent topic addressed in literature. Nevertheless, the high heterogeneity of results with no clear findings makes difficult the understanding of the role of nitrogen compounds, especially amino acids, in *O. oeni* metabolism and MLF performance. In this study, yeast coinoculation resulted the least favourable strategy to enhance MLF. The supplementation with amino acids in initial must promoted MLF in one of the three studied strains - PSU-1 -, which caused stuck fermentation. Still, in *T. delbrueckii* coinoculation with *S. cerevisiae* the supplementation could not enable MLF with PSU-1 but shortened the MLF of the other two *O. oeni* strains in these wines. We were able to relate the nitrogen consumption of *O. oeni* to a decrease in peptide amino acids. The *O. oeni* amino acid consumption pattern was greatly influenced by the yeast inoculation strategy in lower nitrogen content wines (N1), however, this influence was not so clear in wines with higher free amino acids concentration (N2). BA production was highly dependent on the *O. oeni* strain but was not influenced by the amino acid concentration in the must. Cadaverine and putrescine increase after MLF were related with the presence of *odc* gene. In general, the RE of the studied genes responded to the increased amino acid concentration but it did not show an enhanced amino acid metabolism. All the changes observed respond to a highly complex metabolism in *O. oeni*, which is not only dependent on the *O. oeni* strain, but also very dependent on the fermenting yeasts. Future research should address the differences in peptide composition depending on the yeast species/strain and their utilization by *O. oeni*.

Acknowledgements

We thank Sergi Ferrer for kindly providing *O. oeni* Enolab 4783 strain and Laurence Noret for technical support with the HPLC analyses.

Supplementary Figures



Supl. Figure S9. Heat map and clusterization of amino acid concentration (mg N/L). A) Total amino acid concentration of N1 wines. A') Total amino acid concentration of N2 wines. B) Peptide amino acid concentration of N1 wines. B') Peptide amino acid concentration of N2 wines. C) Free amino acid concentration of N1 wines. C') Free amino acid concentration of N2 wines. Increasing colour intensity means higher amino acid consumption. Dendrogram total length correspond to 100% of similarity. Other abbreviations are the same as in Figure 34.

Supplementary Tables

Suppl. Table S11. Amino acid and ammonium composition (mg N/L) of the two musts used in this study*. N1 correspond to the standard concentration found in the grape must. N2 is twice the initial amino acid concentration+.

	N1	N2
Ala	12.9	25.8
Arg	23.4	46.8
Asp	3.2	6.4
Cys	2.3	4.6
Gln	19.7	39.4
Glu	12.4	24.8
Gly	3.3	6.6
His	8.5	17.1
Ile	1.7	3.4
Leu	4.7	9.4
Lys	9.5	19.0
Met	n.d.	n.d.
Phe	3.7	7.4
Ser	5.1	10.3
Thr	3.9	7.3
Trp	3.2	6.4
Tyr	2.4	4.7
Val	3.2	6.5
Pro	360.8	360.8
NH_4	72.2	72.2
Total	556.1	679.2
YAN	195.3	318.4
aa (-Pro)	123.1	246.2

YAN: yeast assimilable nitrogen

* Amino acid and ammonium composition was determined with the method proposed by Gómez-Alonso et al. (2007) as described in **Chapter III: 1**.

+ Proline and NH_4 were not added in N2.

Suppl. Table S12. Primers used in this study.

Locus tag and gene symbol	Sequence (5'→3')	Amplicon length (bp)	Selection criteria	Reference
OEOE_RS08595 (OEOE_1783)	F- TGATCCAGGAGGTCTTCGGT R- TTGGCCGATCCCCATTATCG	203	DEP (Margalef et al., 2016) DEG (Olguín et al., 2015) DEP (**Chapter IV**)	This work
OEOE_RS02110 (OEOE_0438)	F- AGGCCAGGGAAATACAACCG R- GTCGCAATGCCGACGATTAC	186	DEG (Margalef et al., 2016) DEP (**Chapter IV**)	This work
OEOE_RS02735 (OEOE_0574)	F- CTGAGCTGCTAACTGGGCTT R- TGTTTGACCTGGGCACTGTT	128	DEP (Margalef et al., 2016)	This work
OEOE_RS05625 (OEOE_1168)	F- CCGTCATCCTGGTTCAGCTT R- CACCGGCAATCGCTGTAATG	156	DEP (**Chapter IV**) DEG (**Chapter IV**)	This work
OEOE_RS04565 (OEOE_0952)	F- AATGGAAACGGCATGCACAC R- CAAAACCAGGAGTCAAGCGC T	193	DEG (Margalef et al., 2016)	Deltramo et al., 2006
OEOE_RS01985 (OEOE_0413) (*ldhD*)	F- GCCGCAGTAAAGAACTTGATG R- TGCCGACAACACCAACTGTTT	102	Constitutive gene	Desroche et al.,2005
OEOE_RS04805 (OEOE_1000) (*dpolIII*)	F- AATTCGCACGGATTGTTTTC R- GCGAACCAGCATAGGTCAAT	103	Constitutive gene	Stefanelli, 2014
OEOE_RS04780 (OEOE_0995) (*dnaG*)	F- TGTGGACGGAGTGGCAATGT R- CAGTATTTTCTGTATATTTACTATCG	127	Constitutive gene	Desroche et al.,2005
OEOE_ RS00030 (OEOE_0006) (*gyrA*)	F- CGCCCGACAAACCGCATAAA R- CAAGGACTCATAGATTGCCGAA	95	Constitutive gene	Desroche et al.,2005
OEOE_RS00025 (OEOE_0005) (*gyrB*)	F- GAGGATGTCCGAGAAGGAATTA R- ACCTGCTGGGCATCTGTATTG	107	Constitutive gene	Desroche et al.,2005

Suppl. Table S13. General oenological parameters of wines after alcoholic and malolactic fermentation in the two must with standard amino acid concentration (N1) and supplemented with twice the initial concentration (N2). Values shown are the mean of triplicates ± SD. Sc (*S. cerevisiae*), TdB (*T. delbrueckii* Biodiva), TdV (*T. delbrueckii* Viniferm) with C (coinoculation with *S. cerevisiae*) or S (sequentially inoculated with *S. cerevisiae*) refer to the obtained fermented wines. P, 217T and 4783 refer to the MLF strategy where *O. oeni* PSU-1, *O. oeni* 217T, or *O. oeni* Enolab 4783 were inoculated. n.d.: not detected

	L-malic acid (g/L)		pH		Total acidity (g/L)		Volatile acidity (g/L)		Proteins (mg/L)		Ethanol (% vol/vol)	
	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2
Sc	1.92 ± 0.02^{aA}	1.93 ± 0.02^{bA}	3.51 ± 0.06^{deA}	3.38 ± 0.1^{eA}	2.4 ± 0.2^{defA}	2.9 ± 0.3^{bcA}	0.33 ± 0.01^{eA}	0.31 ± 0.02^{gA}	20.8 ± 4.5^{abA}	18 ± 1.2^{bcdA}	11.4 ± 0.6^{aA}	11.8 ± 0.2^{abA}
Sc-P*	0.65 ± 0.04^{eA}	0.08 ± 0.01^{fB}	3.47 ± 0.06^{deA}	3.55 ± 0.01^{abcdA}	2.3 ± 0.1^{efA}	2.5 ± 0^{efghiB}	0.35 ± 0.01^{deA}	0.38 ± 0.02^{deA}	20.6 ± 2.4^{abA}	18.5 ± 5.6^{bcdA}		
Sc-217T	n.d.^{hA}	n.d.^{gA}	3.72 ± 0.01^{abcB}	3.56 ± 0.07^{abcA}	2.3 ± 0.2^{defA}	2.3 ± 0.1^{fghiA}	0.38 ± 0.01^{cdeA}	0.38 ± 0.01^{defA}	20.5 ± 2.7^{abB}	12.3 ± 1.8^{dA}		
Sc-4783	n.d.^{hA}	n.d.^{gA}	3.62 ± 0.18^{bcdA}	3.55 ± 0.01^{abcdA}	2.4 ± 0.1^{efA}	2.3 ± 0.1^{fghiA}	0.36 ± 0.01^{bcdB}	0.34 ± 0.01^{fgA}	16.7 ± 1.1^{abA}	20.4 ± 1.5^{abcdB}		
TdBC	1.52 ± 0.02^{cB}	1.91 ± 0.01^{bA}	3.49 ± 0.1^{deA}	3.42 ± 0^{deA}	3 ± 0.1^{abA}	3.3 ± 0^{aB}	0.36 ± 0.02^{cdeA}	0.39 ± 0.02^{cdeA}	26.4 ± 1.3^{aAA}	24.2 ± 2.7^{abcA}	12 ± 0.6^{aA}	12.3 ± 0.2^{bA}
TdBC-P*	0.6 ± 0.04^{eB}	0.71 ± 0.01^{dA}	3.5 ± 0.17^{deA}	3.51 ± 0.06^{abcdA}	2.6 ± 0.1^{bcdA}	2.5 ± 0.1^{defgA}	0.41 ± 0.01^{abA}	0.42 ± 0.01^{bcA}	25.3 ± 0.7^{aA}	26.5 ± 2.6^{abA}		
TdBC-217T	n.d.^{hA}	n.d.^{gA}	3.57 ± 0.01^{cdeA}	3.55 ± 0.04^{abcdA}	2.5 ± 0.1^{cdeA}	2.4 ± 0^{fghiA}	0.42 ± 0.01^{aA}	0.48 ± 0^{aB}	23.1 ± 1^{abA}	28.6 ± 2.4^{aB}		
TdBC-4783	n.d.^{hA}	n.d.^{gA}	3.54 ± 0.05^{deA}	3.56 ± 0^{abcA}	2.6 ± 0.1^{cdefB}	2.4 ± 0 ^{fghiA}	0.38 ± 0^{bcdA}	0.41 ± 0.01^{bcdB}	22.2 ± 2^{abA}	22.3 ± 3.6^{abcA}		
TdVC	1.54 ± 0.02^{cB}	2.02 ± 0.01^{aA}	3.46 ± 0.02^{eA}	3.47 ± 0.07^{cdeA}	3.1 ± 0.1^{aA}	3.2 ± 0.1^{abA}	0.35 ± 0.02^{deA}	0.35 ± 0.01^{efA}	27.2 ± 3.4^{aB}	18.4 ± 3.8^{bcdA}	12.3 ± 0.2^{aA}	12.4 ± 0.2^{bA}
TdVC-P*	0.86 ± 0.02^{dA}	0.66 ± 0.02^{eB}	3.57 ± 0.02^{cdeA}	3.52 ± 0.04^{abcdA}	2.8 ± 0.2^{abcA}	2.5 ± 0.1^{defA}	0.38 ± 0.02^{abcdA}	0.44 ± 0.01^{bB}	22.5 ± 4.3^{abA}	15.9 ± 2.5^{cdA}		
TdVC-217T	n.d.^{hA}	n.d.^{gA}	3.57 ± 0.01^{cdeA}	3.58 ± 0.03^{abcA}	2.6 ± 0.1^{cdeB}	2.4 ± 0.1 ^{defgA}	0.39 ± 0.01^{abcA}	0.41 ± 0.01^{bcdB}	20.7 ± 3^{abA}	21.2 ± 2.8^{abcdA}		
TdVC-4783	n.d.^{hA}	n.d.^{gA}	3.57 ± 0^{cdeA}	3.6 ± 0^{abcB}	2.6 ± 0.1^{cdA}	2.5 ± 0^{fghA}	0.36 ± 0.01^{cdeA}	0.39 ± 0.01^{cdB}	20.9 ± 3.3^{abA}	24.1 ± 4.1^{abcA}		
TdBS	1.69 ± 0.06^{bA}	1.68 ± 0.01^{cA}	3.5 ± 0.03^{deA}	3.49 ± 0.01^{bcdeA}	2.6 ± 0.1^{bcdA}	2.8 ± 0.1^{cdeA}	0.21 ± 0.02^{hiA}	0.19 ± 0.02^{iA}	18.7 ± 4.5^{ab}	26.1 ± 2.9^{abA}	11.4 ± 0.7^{aA}	11.3 ± 0.3^{aA}
TdBS-P*	0.36 ± 0.02^{fA}	n.d.^{gAB}	3.5 ± 0.01^{bcdeB}	3.64 ± 0^{aA}	2.5 ± 0.1^{defB}	2.1 ± 0^{iA}	0.24 ± 0.02^{ghA}	0.23 ± 0.01^{hA}	24.3 ± 2.2^{abA}	25.8 ± 0.7^{abA}		
TdBS-217T	n.d.^{hA}	n.d.^{gA}	3.71 ± 0.01^{abcB}	3.65 ± 0.01^{aA}	2.3 ± 0.1^{efA}	2.2 ± 0.1^{fghiA}	0.28 ± 0.01^{fB}	0.23 ± 0.03^{hA}	20.5 ± 2^{abA}	20.8 ± 2.9^{abcdA}		
TdBS-4783	n.d.^{hB}	0.07 ± 0.01^{fA}	3.72 ± 0.01^{abcB}	3.64 ± 0^{aA}	2.3 ± 0.1^{efA}	2.3 ± 0.2^{fghiA}	0.22 ± 0.02^{hiB}	0.18 ± 0^{iA}	23.5 ± 2.5^{abA}	28.9 ± 1.9^{aB}		
TdVS	1.71 ± 0.02^{bA}	1.67 ± 0.02^{cA}	3.57 ± 0.03^{cdeB}	3.47 ± 0.04^{cdeA}	2.8 ± 0.1^{abcA}	2.8 ± 0.1^{cdA}	0.2 ± 0.01^{iA}	0.2 ± 0.02^{hiA}	22 ± 1.3^{abA}	25.9 ± 3.4^{abA}	12.2 ± 0.5^{aA}	11.4 ± 0.5^{aA}
TdVS-P	n.d.^{hA}	n.d.^{gA}	3.7 ± 0.01^{abcB}	3.63 ± 0.01^{aA}	2.2 ± 0.1^{f}	2.1 ± 0^{i}	0.27 ± 0.01^{fgB}	0.23 ± 0.01^{hA}	26.8 ± 1.4^{aA}	23.1 ± 4^{abcA}		

TdVS-217T	n.d.^{hA}	n.d.^{gA}	3.79 ± 0.02^{aB}	3.56 ± 0^{abcdA}	2.3 ± 0.1^{ef}	2.2 ± 0.1^{ghi}	0.28 ± 0.01^{fgB}	0.23 ± 0.01^{hA}	19.3 ± 4.3^{abA}	24 ± 1.2^{abcA}
TdVS-4783	n.d.^{hA}	n.d.^{gA}	3.76 ± 0.03^{abB}	3.62 ± 0.01^{abA}	2.3 ± 0.1^{ef}	2.2 ± 0^{hi}	0.22 ± 0.01^{hiB}	0.18 ± 0.01^{iA}	25.1 ± 2.4^{abB}	19.3 ± 0.6^{bcdA}

* Wines with stuck MLF at least in one of the musts.

$^{a–i}$ Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences between values of wines from the same must. Uppercase letters correspond to differences among the values of the inoculation condition from the two musts.

4. General discussion

Since Pasteur, scientific community started to learn about the fermentative process. Nowadays, the microbial agents of alcoholic fermentation (AF) and malolactic fermentation (MLF) are well-known. This knowledge enabled the use of starter cultures to better control the spontaneous fermentative process by using the most adapted microorganisms: *Saccharomyces cerevisiae* for AF, and *Oenococcus oeni* for MLF. In the last two decades the interest in non-conventional yeasts leaded to the investigation of their impact in wine organoleptic profile and fermentative performance (Padilla et al., 2016b). They are usually inoculated together with *S. cerevisiae* to ensure the completion of AF. In this sense, until few years ago, little attention has been paid to the compatibility of MLF in those non-*Saccharomyces* fermented wines (Balmaseda et al., 2018). Thus, the objective of this thesis was to reveal the effects of the two mainly used non-*Saccharomyces* with oenological interest, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, on *O. oeni* and wine MLF.

Even if the nature of interactions between these two microbial groups is still poorly characterised their consequences are a major concern for wine quality. Some aspects in this topic were addressed in this thesis (**Chapter I**). As it usually happens, the fermentative process of different microbial agents is influenced by the medium. Different microbial dynamics in white and red grape musts were observed (**Chapter I: 1**). Still the tendency was similar: the use of *T. delbrueckii* and *M. pulcherrima* extended the duration of AF, while MLF was shortened. The aromatic profile of wines was first modulated by the AF inoculation strategy, but after MLF wines were homogenised according to MLF inoculation strategy. This is interesting to point since the effect of non-*Saccharomyces* was masked by MLF, pointing that the selection of starters of MLF will significantly impact the wine organoleptic profile, somehow regardless the initial volatile composition. The observed improvement in MLF performance was related with a reduction of two well-known antimicrobial compounds as medium chain fatty acids (MCFA) and SO_2. The use non-*Saccharomyces,* mainly *T. delbrueckii,* had a decrease in MCFA concentration by a dramatic reduction of hexanoic acid. Also, *M. pulcherrima* significantly reduced the ethanol content in red wines.

Another important aspect is the polyphenolic composition of red wines. The changing environmental conditions lead to a premature harvest of grapes (Ubeda et al., 2020) where the contribution of wine microbial agents on polyphenolic and aromatic composition is essential. The suitability of some non-*Saccharomyces*, as *T. delbrueckii* for red winemaking, has been already reported in terms of aroma and polyphenolic profile (Benito, 2018a; Escribano-Viana et al., 2019; Escribano et al., 2018). Nevertheless, the effects of that chemical modulation towards *O. oeni* were still unclear. Polyphenolic compounds, which are a distinctive attribute of red wine, may affect *O. oeni* viability (García-Ruiz et al., 2011; Reguant et al., 2000), and thus, affect MLF performance. It was studied the *T. delbrueckii* positive impact with red grapes to evaluate the compatibility of this yeast species together with *O. oeni* in terms of polyphenolic composition and MLF performance (**Chapter I: 2**). *T. delbrueckii* enhanced the polyphenolic composition of wines, mainly due to an increased anthocyanin concentration, and also promoted MLF. This was an interesting fact since, as introduced before, high polyphenolic wines are related with inhibition of *O. oeni* fermentative process. There should be other compounds, different from those determined in this study, derived from *T. delbrueckii* metabolism, which can promote MLF and help to mitigate the negative effects of polyphenols on *O. oeni*, as also observed in **Chapter I: 1**.

The opportunity of working with natural musts under cellar conditions, mimicking a real fermentative process, with natural occurring microbiota, enabled to better understand the microbial dynamics, especially *O. oeni* population. Two *O. oeni* strains (PSU-1 and CH11) were used and also spontaneous MLF, without inoculation, was evaluated. The imposition of inoculated strains at the end of MLF was different. In this sense, PSU-1 showed higher imposition rate than CH11. Besides, the use of non-*Saccharomyces* modulated the *O. oeni* autochthonous population affecting significantly to MLF performance and duration (**Chapter I: 1, 2**). Higher diversity was observed in wines described in **Chapter I: 1** possibly due to less restrictive conditions as low ethanol content and polyphenols and moderate pH values. On the contrary, wines from **Chapter I: 2** with high ethanol and polyphenolic contents, coming from the more mature grapes, showed less biodiversity. Still, the number of different strains detected increased as result of the use of *T. delbrueckii*. Moreover, the use of this

particular yeast was responsible of the development of the spontaneous MLF that in wines with *S. cerevisiae* as sole starter did not occur.

Then, and considering *T. delbrueckii* as the main focus of the thesis, it was tried to further characterise the population modulation on a more controlled essay. Apart from the work of Tantikachornkiat et al. (2020), which studied the microbial strain dominance at the end of AF and MLF, there were no other studies considering the effect of AF starters in MLF population. A synthetic *O. oeni* community was designed with five different strains and a sterile must was inoculated in a population similar to the found in nature (**Chapter III**). Four of these strains were isolated from wines fermented with *T. delbrueckii* in **Chapter II: 1** and **Chapter II: 2**. Under this controlled condition, the *O. oeni* community responded differently in wines fermented with *T. delbrueckii* or *S. cerevisiae*. Besides, strain specific compatibility was observed. It is worth noting that the initial *O. oeni* population was maintained in *T. delbrueckii* wines until *S. cerevisiae* was inoculated, probably due to the high fermentative capacity exhibited in this medium by *S. cerevisiae* with respect to *T. delbrueckii*. Using two *T. delbrueckii* strains in sequential inoculation with *S. cerevisiae*, wines fermented with *T. delbrueckii* Biodiva presented high similarity to the population of *S. cerevisiae* wine. Also, fermentation parameters were modified according to the AF inoculation strategy. Considering just the duration of exponential consumption of L-malic acid by *O. oeni*, a significant reduction was observed in *T. delbrueckii* wines with respect to *S. cerevisiae* ones. Thus, even if the *O. oeni* strain population was different, a general stimulation was observed using *T. delbrueckii*. As it exists a strain-specific compatibility between yeasts and *O. oeni* strains, it should be carefully selected those compatible tandems to use them as starters in winemaking. Indeed, as previously reported, the use of certain AF inoculation strategies will impact on the composition of *O. oeni* population and determine the imposition of the inoculated *O. oeni* strain starter. Considering this, it would be possible to favour the imposition of a particular *O. oeni* strain with the most suitable metabolism for the wine organoleptic profile we want to produce.

The compatibility between yeasts and *O. oeni* starters can help or even improve MLF performance. In this sense, the survival of *O. oeni* is also crucial. *O. oeni* is a fastidious bacterium which has to develop in a very impoverished medium with low

nutrient availability (Terrade and Mira de Orduña, 2009). Traditionally, MLF has been performed under the presence of yeast lees and it has been associated with an improvement in MLF performance and *O. oeni* survival (Guilloux-Benatier et al., 1995). After AF, wine yeasts – yeast lees - begin to lose viability and they suffer eventually an autolytic process that releases some of their intracellular components together with other yeast wall related compounds (Alexandre and Guilloux-Benatier, 2006).

In this new winemaking context, where the use of non-*Saccharomyces* yeasts begins to increase, the characterisation of the effect of these different yeasts as lees appears as an important issue for the MLF. The different metabolisms and peculiarities of each yeast species, or even strains, will modify the released compounds during the autolytic process. In this sense we performed MLFs under lees of different strains of *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* (**Chapter III: 1**). A synthetic wine (wine-like medium, WLM) with defined composition, where those different lees were added separately, was inoculated with three *O. oeni* strains. Contrary to the general knowledge, the addition of these lees did not always had a positive effect in the fermentative parameters of MLF, also reported by other authors (Herrero et al., 2003; Patynowski et al., 2002). The behaviour was very strain dependent regarding the effect of the inoculated yeasts lees; from the three studied *O. oeni* strains, CH11 improved its MLF performance, PSU-1 performance was very dependent on the yeast lees strain, and 1Pw13 was, in general, negatively affected by the addition of yeast lees. In this way, the compatibility of yeast lees with *O. oeni* seems to be strain specific as observed previously in the fermenting yeast - *O. oeni* strain compatibility (**Chapter II**). The amino acid consumption by *O. oeni* together with other oenological related parameters was evaluated and high heterogeneity of results were obtained. This manifests a complex compatibility patterns that should be considered. Still, the best MLF performance for all the strains were obtained in wines supplemented with *T. delbrueckii* yeast lees. Their addition produced an increase in pH value and higher mannoprotein concentration in wine.

Probably the main compounds derived from yeast lees related with a stimulatory effect upon *O. oeni* are mannoproteins. These macromolecules are polysaccharides of mainly mannose and glucose, with some residues of amino acids (Giovani et al., 2012).

Interestingly, *O. oeni* can breakdown these compounds by an extracellular peptidase activity and incorporate mannose residues or even littler peptides or amino acids. In **Chapter III: 1** a decrease in mannoprotein concentration was shown under oenological conditions, and thus, a possible utilisation and consumption by *O. oeni* during MLF. Indeed, in some cases, the use of these macromolecules was related with an improvement in MLF (**Chapter III: 1**). To better address this phenomenon, mannoprotein utilisation was deeper evaluated and some genes related with the use of mannose uptake in *O. oeni* were also considered (**Chapter III: 2**). The consumption and relative expression of those genes in WLM supplemented with a commercial mannoprotein extract in increasing concentration was evaluated. The selected four genes were those encoding for two mannose permeases (*manA* and *manB*) and two general proteins (*ptsI* and *ptsH*) of the phosphoenolpyruvate phosphotransferase system (PTS) responsible of the mannose uptake and utilisation (Jamal et al., 2013).

The mannoprotein utilisation was concentration dependent. Higher consumption was observed when the mannoprotein availability was increased. The use of alternative energy sources seems to be activated when L-malic acid is depleted, as it occurs with citric acid (Bartowsky and Henschke, 2004). In this sense, we also measured extracellular mannoprotein concentration three days after the completion of MLF and observed a dramatic decrease of mannoproteins. Observing the relative expression (RE) of the selected genes, there was a general increase in their transcriptional levels during the fermentative process, being the highest RE in post-MLF point. The four genes were upregulated under oenological conditions but only *manB* and *ptsI* were activated by increased concentration of mannoproteins in the media. The general activation of these genes is due to a non-specific response to hexoses (Jamal et al., 2013). Nevertheless, *manA* and *manB* (apart from *manC* which another gene of a mannose permease but not highly conserved within *O. oeni* strain genomes), encode for mannose permeases in *O. oeni* which are not selective to other hexoses. Besides, as they respond to wine-like conditions, the study of mannose uptake throughout the study of their transcriptional levels give not much information about how *O. oeni* respond to mannose or mannoprotein levels.

The mannoprotein utilisation and RE of those genes in non-*Saccharomyces* fermented wines were also studied (**Chapter III: 2**). These wines were fermented with a mannoprotein overproducer *S. cerevisiae* strain (Belda et al., 2016) and with two *T. delbrueckii* strains sequentially inoculated with a standard *S. cerevisiae*. In these wines the RE of the genes in *O. oeni* was calculated comparing to the control wine fermented with *S. cerevisiae* QA23. The *manA* levels were similar in all wines and the expression of *manB*, *ptsI*, and *pstH* were upregulated when using non-*Saccharomyces*. On the contrary, the mannoprotein utilisation was not increased. Thus, mannose related genes in *O. oeni* are more active in non-*Saccharomyces* wines, which can be an advantage for the bacterium to use alternative energy sources in wine, even if we could not relate it with an enhanced mannoprotein utilisation in our study.

The changes produced by the use of non-*Saccharomyces* in wine seems to have an impact on *O. oeni* molecular mechanisms since its behaviour is different in those wines. Besides, the transcriptional levels of some *O. oeni* genes related with mannose uptake were modified in wines fermented with different yeasts (**Chapter III: 2**). There is a large number of molecular mechanisms that *O. oeni* activates in wine-like conditions in order to survive. As result, there are large number of genes and proteins modified when *O. oeni* is under oenological conditions comparing to the preculture in laboratory medium. Some of them are related with ethanol shock (Olguín et al., 2015), others with acidic shock (Liu et al., 2017a), but globally there is a very complex adaptation response to wine (Margalef-Català et al., 2016a). To identify the adapted molecular mechanisms by the use of *T. delbrueckii* and *M. pulcherrima*, AFs with those yeasts were performed in sequential inoculation and also a control fermentation with *S. cerevisiae*. Then, *O. oeni* PSU-1, strain with the genome completely sequenced (Mills et al., 2005), was inoculated in each wine and its adaptation during MLF in each wine was compared. For that purpose, Ion Torrent RNA sequencing technology and quantitative proteomics with isobaric labelling and nanoLC-MS/MS were used to study the transcriptional and proteomic changes (**Chapter IV**).

The adaptation mechanisms observed were dependent on the yeast strain combination. Indeed, differences in MLF duration were observed. Clearly, the use of non-*Saccharomyces* reduced the duration of the fermentative process, from the half in the case of *T. delbrueckii* and to a quarter with *M. pulcherrima*. It is worth noting that

the number of differentially expressed genes (DEG) and proteins (DEP) in *O. oeni* adaptation during MLF was considerable. Margalef et al. (2016) identified 622 DEG, and 33 to 71 DEP, depending on the analytic technique. The high number of DEG detected in that work respond to the dramatic medium change, which is the inoculation of *O. oeni* in wine-like medium from a rich culture medium. In the present work, and considering the adaptation process, which was defined as the molecular changes at the end of MLF respect to the inoculation time (1 h after the inoculation in wine), less changes were observed, mainly in DEG. 66, 69 and 101 DEG (excluding tRNAs) were detected in *O. oeni* fermenting in *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation wines, respectively. Similar DEP were detected: 82, 57, and 100 DEP in *O. oeni* fermenting in *S. cerevisiae*, *T. delbrueckii* sequential inoculation, and *M. pulcherrima* sequential inoculation wines, respectively. In this sense, the use of more advanced proteomic analyses allowed to identify large number of DEP. Interestingly, the abundance of the malolactic enzyme was similar in all wines, and thus, it could not explain the different durations of MLF. That is why the other observed changes (DEG and DEP) should be responsible for the different MLF duration in non-*Saccharomyces* wines. For all these DEG and DEP, the main metabolisms affected were amino acid and carbohydrate transport and metabolisms. From these metabolisms, it is interesting to mention that the main changes observed in amino acid metabolism were related with genes encoding for peptidases and peptide transport proteins. Even if *O. oeni* exhibits low nitrogen demand, it is usually related with peptide utilisation (Remize et al., 2006). In this study it was confirmed that the expression of genes related with peptide utilisation was increased in non-*Saccharomyces* fermented wines and could be related with that improvement of MLF performance. Contrary to other studies focused on stress response in *O. oeni* to wine-like conditions, low DEG and DEP related with stress response were detected. Thus, the general stress to wine-like conditions remained similar in the three wines and it was not dependent on the AF inoculation strategy. Besides, the abundance of a well-known heat shock protein (Hsp20) was increased as the duration of MLF increased. Indeed, it has been proposed as stress response marker (Coucheney et al., 2005b; Olguín et al., 2010). In this sense, a decrease in Hsp20 in

non-*Saccharomyces* wines corresponded to a less stressful wine-like conditions for *O. oeni* and confirms the positive effect of non-*Saccharomyces* in wine.

One of the main metabolisms found differently regulated in *O. oeni* fermenting in non-*Saccharomyces* wines was amino acid transport and metabolism (**Chapter IV**), particularly related with the use of peptides. The nitrogen demand of *O. oeni* in wine is not very well-known due to the high heterogeneity of results obtained in wine conditions. It is usually addressed regarding to the amino acid preferences of the bacterium in growth medium (Aredes Fernández and Manca De Nadra, 2006). Besides, there are some works that point out the great importance of peptides as nitrogen source for *O. oeni* in wine (Remize et al., 2006). Related with nitrogen metabolism, the production of biogenic amines (BA) is also a very important aspect. These compounds are the result of the amino acid decarboxylation, and they are reported to be toxic to humans. That is why one of the main criteria in *O. oeni* starter selection is the absence of BAs production.

Further, in **Chapter V** the nitrogen metabolism of three *O. oeni* strains in *T. delbrueckii* fermented wines (coinoculated and sequentially inoculated with *S. cerevisiae*) was studied in comparison to *S. cerevisiae* wine. In addition, two musts, one of them with twice the initial amino acid concentration, were used. In general, the supplementation with amino acids did not improve MLF performance. Nevertheless, it had a strong effect on PSU-1 strain, which produced stuck fermentation in the standard must. The supplementation allowed PSU-1 to finish MLF in all wines except for coinoculated wines. Indeed, coinoculation with non-*Saccharomyces* and *S. cerevisiae* seems to produce some compounds, still not characterised, that produce a very harsh medium for *O. oeni* (Martín-García et al., 2020). This is probably related with high competition between the two yeast strains. The concentration of free amino acids and peptides was quantified before and after MLF. In this sense, the *O. oeni*'s preference of peptides as nitrogen source was confirmed. In all wines, the peptide amino acid concentration decreased after MLF, while free amino acid concentration remained similar. The peptide composition of wines inoculated with different AF strategies was similar in concentration but not in composition. It is worth noting that there are no studies about the characterisation of peptide composition in wine by different yeasts. Thus, the characterisation of them should be further investigated in

order to generate new knowledge, and also to better understand nitrogen metabolism of *O. oeni* in wine.

In **Chapter V** it was also studied the transcriptional response of some genes related with amino acid transport and metabolism that showed differential expression in **Chapter IV** and other studies (Margalef-Català et al., 2016a; Olguín et al., 2010) in *O. oeni* PSU-1. These genes encode for peptidases, transporters and for the glutamine synthase, and all them showed an increased transcriptional level in response to *T. delbrueckii* and to the increasing initial amino acid concentration. Nevertheless, the increased expression did not guarantee finishing of MLF by *O. oeni* PSU-1. The production of BA did not depend on the availability of each amino acid precursor. It was more related with the fermenting medium and was different for each BA quantified. Particularly, the production of putrescine and cadaverine was confirmed to be related with the presence of the gene *odc*. Significant high concentrations of these BAs were quantified in the wines fermented with *O. oeni* Enolab 4783, which was the only of the used strains with the *odc* gene. In this sense, some ornithine decarboxylases (ODC) not only can use ornithine and produce putrescine, but also lysine to produce cadaverine (Romano et al., 2012). Thus, the increased cadaverine content in Enolab 4783 fermented wines should be related with a non-specific substrate ODC enzyme. 2-phenylethylamine was not related with MLF. Interestingly, this BA increased only by AF and the lowest concentrations were quantified in sequential inoculation with *T. delbrueckii*.

In summary, this thesis has been focused on the effects of *T. delbrueckii* and *M. pulcherrima* on *O. oeni* and MLF. Throughout this work different oenological effects were addressed. It was highlighted the importance of the yeast - *O. oeni* strain combinations to enhance some oenological attributes. Moreover, the use of particular yeast strains can also modulate the fermenting *O. oeni* population in wine. One of the stimulatory effects observed in non-*Saccharomyces* wines was the reduction of some inhibitory wine compounds as MCFA, ethanol and SO_2. It was also confirmed by the comparative omic approach that *O. oeni* responds differently in non-*Saccharomyces* fermented wines. This is very important since apart from the highly complex stress response to wine-like conditions (Margalef-Català et al., 2016a), the molecular adaptation of *O. oeni* varies in each wine, even if not many stress response DEG/DEP

were detected. In this sense, the general statement of the beneficial use of non-*Saccharomyces* on *O. oeni* (Balmaseda et al., 2018) was confirmed by a less abundance of Hsp20 regarding to *S. cerevisiae* fermented wine. From those molecular mechanisms differentially expressed in non-*Saccharomyces* wines, carbohydrate and amino acid metabolisms are standing out. According to carbohydrates, the metabolism of mannose, coming from mannoproteins, was also stated as a useful metabolism in *O. oeni* under wine-like conditions, which was enhanced in increased mannoprotein concentrations and complex media. According to amino acid metabolism, amino acids from peptides were also confirmed as key nitrogen compound for *O. oeni* in wine. Further research in these two metabolisms would help to better understand the positive effects observed in this thesis in relation to the use of *T. delbrueckii* and *M. pulcherrima*. To conclude, the results obtained bring out the potential of non-*Saccharomyces* in stimulating MLF in harsh conditions, and also modulating wine organoleptic attributes.

5. Conclusions

The main conclusions obtained from this thesis are:

1. The use of *T. delbrueckii* and *M. pulcherrima* modulates the chemical composition in wine. They can reduce the concentration of inhibitory compounds as ethanol, MCFA and SO_2. Also, they contribute to enhance the aroma profile and polyphenolic composition.

2. Wine fermentation dynamics and population are affected by non-*Saccharomyces*. The AF duration is extended while MLF duration is shortened. Besides, *O. oeni* population diversity is increased in non-*Saccharomyces* fermented wines, and *T. delbrueckii* can promote MLF in harsh conditions.

3. The effect of yeast lees is dependent on the yeast species and the specific interaction with the fermenting *O. oeni* strain, ranging from stimulatory, to neutral or inhibitory. In this sense, *T. delbrueckii* lees are more related to a stimulatory effect.

4. Mannoprotein utilization is a stress response metabolism in *O. oeni* under wine conditions. It is active in wine conditions and responds to increased mannoprotein content and late MLF stages.

5. *O. oeni* molecular mechanisms are adapted to non-*Saccharomyces* fermented wines. From those, amino acid and carbohydrate metabolisms are the most altered ones.

6. The use of non-*Saccharomyces* can contribute to a decreased wine stress state in *O. oeni* related with a less abundance of the stress protein Hsp20.

7. Nitrogen metabolism has a complex regulation in *O. oeni*, dependent on the medium, where peptides are the preferent nitrogen source in wine. Indeed, peptide utilization related genes are activated in wine conditions.

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Annex I

Publications derived from this thesis

Publications in indexed journals

Balmaseda A, Bordons A, Reguant C, Bautista-Gallego J* (2018). Non-*Saccharomyces* in wine: Effect upon *Oenococcus oeni* and malolactic fermentation. *Frontiers in Microbiology* 9, 534, 1-8. https://doi.org/10.3389/fmicb.2018.00534

Martín-García A, **Balmaseda A**, Bordons A*, Reguant C (2020) Effect of the inoculation strategy of non-*Saccharomyces* yeasts on wine malolactic fermentation. *Oeno One* 54, 1, 101-108. https://doi.org/10.20870/oeno-one.2020.54.1.2906

Balmaseda A, Rozès N, Leal MA, Bordons A, Reguant C* (2021) Impact of changes in wine composition produced by non-*Saccharomyces* on malolactic fermentation. *Int J. Food Microbiol.* 337, 108954. https://doi.org/10.1016/j.ijfoodmicro.2020.108954

Balmaseda A, Rozès N, Leal MA, Bordons A, Reguant C* (2021). *Torulaspora delbrueckii* promotes malolactic fermentation in high polyphenolic red wines. *LWT - Food Sci. Technol.* 148, 111777 https://doi.org/10.1016/j.lwt.2021.111777

Balmaseda A, Rozès N, Leal MA, Bordons A, Reguant C* (2021). Simulated lees of different yeast species modify the performance of malolactic fermentation by *Oenococcus oeni* in wine-like medium. *Food Microbiology. 99, 103839* https://doi.org/10.1016/j.fm.2021.103839

Other publications

Reguant C, **Balmaseda A**, Ferrando N, Bordons A (2019). Impacto del uso de levaduras no-Saccharomyces sobre la fermentación maloláctica del vino. *Revista Alimentaria* 502, 28-30

Balmaseda A, Martín-García A, Leal MA, Rozès N, Bordons A, Reguant C (2021) Non-*Saccharomyces* as a tool for modulating wine quality and stimulating malolactic fermentation. IVES Technical Reviews Vine & Wine, 9 April 2021. https://ives-technicalreviews.eu/article/view/4661

Annex II

Non-*Saccharomyces* in wine: effect upon *Oenococcus oeni* and malolactic fermentation

frontiers in Microbiology

MINI REVIEW
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Non-*Saccharomyces* in Wine: Effect Upon *Oenococcus oeni* and Malolactic Fermentation

*Aitor Balmaseda[1], Albert Bordons[1], Cristina Reguant[1] and Joaquín Bautista-Gallego[2]**

[1] Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, Catalonia, Spain,
[2] Food Biotechnology Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Universidad Pablo de Olavide, Seville, Spain

This work is a short review of the interactions between oenological yeasts and lactic acid bacteria (LAB), especially *Oenococcus oeni*, the main species carrying out the malolactic fermentation (MLF). The emphasis has been placed on non-*Saccharomyces* effects due to their recent increased interest in winemaking. Those interactions are variable, ranging from inhibitory, to neutral and stimulatory and are mediated by some known compounds, which will be discussed. One phenomena responsible of inhibitory interactions is the media exhaustion by yeasts, and particularly a decrease in L-malic acid by some non-*Saccharomyces*. Clearly ethanol is the main inhibitory compound of LAB produced by *S. cerevisiae*, but non-*Saccharomyces* can be used to decrease it. Sulfur dioxide and medium chain fatty acids (MCFAs) produced by yeasts can exhibit inhibitory effect upon LAB or even result lethal. Interestingly mixed fermentations with non-*Saccharomyces* present less MCFA concentration. Among organic acids derived as result of yeast metabolism, succinic acid seems to be the most related with MLF inhibition. Several protein factors produced by *S. cerevisiae* inhibiting *O. oeni* have been described, but they have not been studied in non-*Saccharomyces*. According to the stimulatory effects, the use of non-*Saccharomyces* can increase the concentration of favorable mediators such as citric acid, pyruvic acid, or other compounds derived of yeast autolysis such as peptides, glucans, or mannoproteins. The emergence of non-*Saccharomyces* in winemaking present a new scenario in which MLF has to take place. For this reason, new tools and approaches should be explored to better understand this new winemaking context.

Keywords: non-*Saccharomyces*, malolactic fermentation, *Oenococcus*, wine, microbial interactions

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Vittorio Capozzi,
University of Foggia, Italy

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Lucía González-Arenzana,
Instituto de Ciencias de la Vid y del Vino (ICVV), Spain
Gianluca Bleve,
ISPA-CNR, Italy

****Correspondence:***
Joaquín Bautista-Gallego
joaquinbg@ig.csic.es

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INTRODUCTION

Wine is the result of the alcoholic fermentation (AF) driven out by oenological yeasts in a complex microbial environment (Constantí et al., 1997; Beltran et al., 2002). Apart from *Saccharomyces cerevisiae*, recognized as the main agent of this process, other yeast species, known as non-*Saccharomyces* yeasts, such as *Hanseniaspora/Kloeckera*, *Pichia*, *Candida*, or *Metschnikowia* are implicated in early stages of the AF (Fleet et al., 1984). After the AF, the resultant wine can undergo the malolactic fermentation (MLF), which consists on a fairly simple reaction: a unique enzymatic decarboxylation of the L-malic acid to L-lactic acid (Liu, 2002). It is usually performed

in red wines or high acidity white wines. This fermentation is carried out by lactic acid bacteria (LAB). Four LAB genera are usually found in wine: *Lactobacillus*, *Pediococcus*, *Leuconostoc,* and *Oenococcus;* and particularly, the main dominant species in wine is *Oenococcus oeni* (Wibowo et al., 1985; Lonvaud-Funel, 1999; Liu, 2002). MLF is related to a quality improvement in wine since this biotransformation leads to a pH increase, enhanced organoleptic properties and a microbial stabilization (Lonvaud-Funel, 1999). During MLF, LAB consume L-malic acid and other nutrients, impoverishing wine and avoiding the development of contaminant microorganisms.

In the last few years the interest on the use of non-*Saccharomyces* yeasts in winemaking has increased (Padilla et al., 2016; Petruzzi et al., 2017), due to the particular enzymatic activities that catalyze the liberation of aromas from their non-volatile precursors (Belda et al., 2017). Generally, these yeasts are inoculated to start the AF of must and later *S. cerevisiae* is inoculated to finish the process. This type of sequential inoculation with non-*Saccharomyces* undergoes chemical changes in wine which modulate the organoleptic profile of wines (Fleet, 2008; Padilla et al., 2016). What is more, this chemical modulation presents new scenery in which MLF may take place.

The purpose of this mini review is to summarize the current knowledge about the compounds responsible for the interactions that may take place between oenological yeasts and LAB during winemaking, highlighting the new scenery of non-*Saccharomyces* fermentations.

YEAST-LAB INTERACTIONS: OENOLOGICAL CONTEXT

The performance of MLF by LAB is highly affected by the physicochemical intrinsic properties of wine, such as pH, ethanol, and SO_2 (Carreté et al., 2002; Arnink and Henick-Kling, 2005). Moreover, since MLF takes place usually after the AF, it is also influenced by yeast metabolism. Those interactions range from inhibitory, to neutral and stimulatory. There is not much literature about this topic, but it is agreed that the type and impact of the interactions is dependent on several factors like (I) the initial must composition, (II) the yeast/bacteria strain combination, (III) the uptake and release of nutrients by yeasts, and (IV) the ability of yeasts to produce metabolites that affect somehow LAB (King and Beelman, 1986; Lonvaud-Funel et al., 1988; Alexandre et al., 2004; Du Plessis et al., 2017). There are some compounds which mediate these interactions (**Figure 1**) but, still the available information is not sufficient.

Up to date, some strategies have been developed to mitigate the possible yeast- *O. oeni* inhibitory interactions (Sumby et al., 2014). Specifically, coinoculation of yeast and *O. oeni* has been proposed as a promising strategy to reduce the length of MLF (Izquierdo Cañas et al., 2014). In this way, the simultaneous AF and MLF co-immobilized in alginate beads is a technique currently in study (Bleve et al., 2016). Another classical approach to deal with the MLF difficulties is to select specific strains from the nature (Campbell-Sills et al., 2017; Petruzzi et al., 2017). The purpose of this selection is to identify the most relevant microorganisms related with the fermentation process in a particular area and use them as culture starters (Portillo et al., 2016; Franquès et al., 2017; Petruzzi et al., 2017).

Above the direct yeasts effect upon LAB and MLF performance, the must, and the winemaking practices, have a strong impact in how these interactions take place (Arnink and Henick-Kling, 2005; Tristezza et al., 2016).

Beyond the particular production of certain compounds (**Table 1**), yeast metabolism exhausts the nutrients of the medium. LAB have complex nutrient requirements (Garvie, 1967; Fourcassie et al., 1992; Terrade and Mira de Orduña, 2009), so their growth is highly dependent on the nutrients consumption during AF by yeasts (Ivey et al., 2013). The effect of these inhibitory interactions could be explained as the result of nutrient competition, such as yeast assimilable nitrogen (YAN) or amino acids (Costello et al., 2003). Therefore, yeast strains with complex nutrient requirements would exhibit an increased antagonistic relationship with LAB (Costello et al., 2003). In this way, it has been recently described that coinoculation of *S. cerevisiae* with other non-*Saccharomyces* yeasts result in a metabolic stimulation of glucose and nitrogen uptake by yeasts, which could lead to a more impoverished medium for LAB (Curiel et al., 2017).

Moreover, it has been reported that the use of some yeast strains (Su et al., 2014) can cause a decrease in L-malic acid, the prior substrate of LAB in wine, which can negatively affect the MLF performance. Particularly, the use of non-*Saccharomyces* leads a higher consumption of L-malic acid, as it has been described with *Torulaspora delbrueckii* (Belda et al., 2015), *Starmerella bacillaris* (syn. *Candida zemplinina*) (Tofalo et al., 2012; Du Plessis et al., 2017), *M. pulcherrima* (Du Plessis et al., 2017), and *Issatchenkia orientalis* (Kim et al., 2008). There is also another non-*Saccharomyces* yeast that really consumes L-malic acid to dryness (Du Plessis et al., 2017). *Schizosaccharomyces* spp. can develop the maloalcoholic fermentation by consuming both sugars and L-malic acid (Benito et al., 2013, 2014).

Alcoholic fermentation of grape must undergoes deep chemical changes enhanced by ethanol and sulfur dioxide. Long ago, it is agreed that concentrations over 4% (v/v) of ethanol inhibit the growth of most LAB (Capucho and San Romao, 1994). Also, a more recent study reported the triad of ethanol, SO_2 and medium chain fatty acids (MCFAs) as the main inhibitor compounds in the antagonism between yeast and *O. oeni* (Nehme et al., 2008). The main functional categories of genes affected by ethanol are metabolite transport and cell wall and membrane biogenesis (Olguín et al., 2015). Nowadays, some non-*Saccharomyces* yeasts are currently used in mixed fermentations to decrease the alcoholic content of wines (Giaramida et al., 2013; Loira et al., 2014; Ciani et al., 2016), such as *M. pulcherrima* (Contreras et al., 2014), *T. delbrueckii* (Belda et al., 2015), *C. stellata* (Ferraro et al., 2000) and *S. bacillaris* (Englezos et al., 2016a), possibly mitigating the negative effect of ethanol upon LAB growth.

The role of SO_2 as an antimicrobial compound is known since ancient Romans that used to add this chemical to prevent food and beverage from spoilage. Its active mechanism affects *O. oeni* membrane and causes an ATPase activity decrease

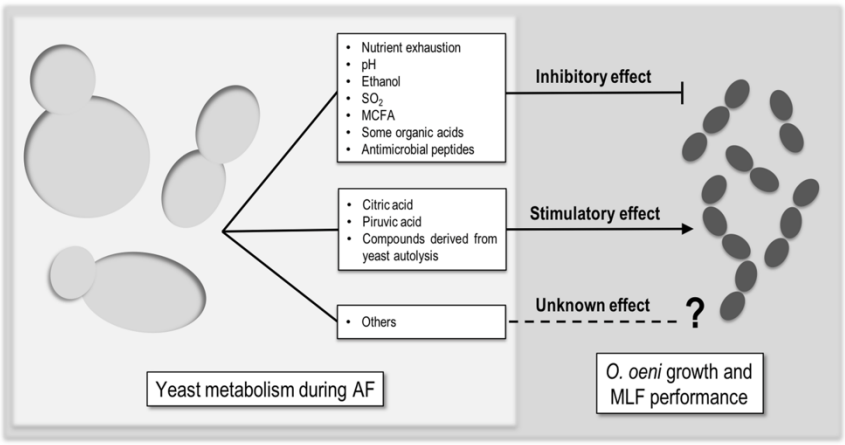

FIGURE 1 | Compounds produced by yeast that can mediate inhibitory, stimulatory, or unknown effect in *Oenococcus oeni* growth or MLF performance.

(Carreté et al., 2002), causing a delay or the failure of MLF (Lonvaud-Funel et al., 1988). It is customary to use this compound to control microbial communities since vineyard to wine in the winemaking. Moreover, yeasts are able to produce this compound as result of their metabolism (Wells and Osborne, 2011). The common amount of SO_2 produced by *S. cerevisiae* strains is less than 30 mg/L, but some strains can produce more than 100 mg/L of this compound (Suzzi et al., 1985; Rankine, 1968). When it comes to non-*Saccharomyces* yeasts, there is no much information about their SO_2 production since they are more affected by this compound (Jolly et al., 2014). However, it has to be pointed that the use of *T. delbrueckii* as sole starter increased the SO_2 concentration of the final wine (Belda et al., 2015). Apart from the cited strain effect, the medium has great influence in the production of SO_2 by yeasts. Higher concentration of YAN in must ends on higher amount of SO_2 (Osborne and Edwards, 2006), as result of the metabolism of the sulfured amino acids.

Medium Chain Fatty Acids (MCFAs)

During AF, yeasts produce different compounds as result of their growth metabolism that can inhibit *O. oeni* growth and MLF. MCFA (C_8–C_{14}) constitute a group of organic molecules that can limit *O. oeni* growth and even decrease their L-malic consumption (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988). It has to be mentioned the strong effect of winemaking practices in fatty acids metabolism by yeasts (Guilloux-Benatier et al., 1998). These authors related a fine MLF performance with a large pre fermentative maceration, possibly due to the high macromolecules concentration and long chain fatty acid extraction (Guilloux-Benatier et al., 1995, 1998). The effect of using non-*Saccharomyces* yeasts in the production of MCFA is variable. Strains belonging to *M. pulcherrima*, *C. stella,* and *Pichia fermentans* increase the final concentration of MCFA (Liu P.-T. et al., 2016). In contrast, mixed fermentations with *H. uvarum*, *I. orientalis* present the opposite behavior (Liu P.-T. et al., 2016). Also, a significant decrease in MCFA concentration has been reported by *Lanchacea thermotolerans* as sole starter (Shekhawat et al., 2017). Hu et al. (2018) reported a strong influence in MCFA concentration related with the inoculation timing of *H. uvarum* in mixed fermentation with *S. cerevisiae*. In this experiment inoculation timing seem to determine the increase or decrease in MCFA concentration regarding to *S. cerevisiae* traditional fermentation. Generally, C_{12} and C_{14}, as free fatty acids, are the most toxic MCFA for *O. oeni* (Guilloux-Benatier et al., 1998). Moreover, the esterified forms are even more toxic than free fatty acids, being the most toxic esterified MCFA C_{10}, C_{12}, and C_{14} (Guilloux-Benatier et al., 1998). So, depending on the particular MCFA and its concentration, the inhibitory effect can become lethal to LAB (Edwards and Beelman, 1987).

Organic Acids Similar to L-Malic Acid

Malolactic fermentation is the consequence of a unique enzymatic activity performed by the malolactic enzyme. Accordingly, structurally similar organic acids will act as competitive inhibitors for the active site of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982) and probably they will delay the MLF duration. Early studies in this subject related this effect with succinic acid, fumaric acid, citric acid, and tartaric acid (Lonvaud-Funel and Strasser de Saad, 1982; Davis et al., 1985). Among these acids, succinic acid is the most studied since oenological yeasts can largely produce this compound. First studies related the inhibition of MLF by criotolerant *S. cerevisiae* strains which are characterized by high production of succinic

TABLE 1 | Main compounds affected (variation in content, negative or positive) by the use of non-*Saccharomyces* in alcoholic fermentation regarding to *S. cerevisiae* as sole starter.

Compound	Non-*Saccharomyces**	Variation respect to *S. cerevisiae*	Reference
L-Malic acid	*T. delbrueckii* + *S. c.*	–	Belda et al., 2015
	S. bacillaris + *S. c.*	–	Tofalo et al., 2012; Du Plessis et al., 2017
	M. pulcherrima + *S. c.*	–	Du Plessis et al., 2017
	I. orientalis + *S. c.*	–	Kim et al., 2008
	Sc. pombe + *S. c.*	–	Benito et al., 2013, 2014
Ethanol	*M. pulcherrima* + *S. c.*	–	Contreras et al., 2014
	T. delbrueckii + *S. c.*	–	Belda et al., 2015
	C. stellata + *S. c.*	–	Ferraro et al., 2000
	S. bacillaris + *S. c.*	–	Masneuf-Pomarede et al., 2015
Sulfur dioxide	*T. delbrueckii*	+	Belda et al., 2015
Medium chain fatty acids	*H. uvarum* + *S. c.*	–	Liu P.-T. et al., 2016
	I. orientalis + *S. c.*	–	Liu P.-T. et al., 2016
	T. delbrueckii + *S. c.*	–	Belda et al., 2015
	L. thermotolerans	–	Shekhawat et al., 2017
	M. pulcherrima + *S. c.*	+	González-Royo et al., 2015; Liu P.-T. et al., 2016
	C. stella + *S. c.*	+	Liu P.-T. et al., 2016
	P. fermentans + *S. c.*	+	Liu P.-T. et al., 2016
Citric acid	*S. bacillaris* + *S. c.*	+	Giaramida et al., 2013
Pyruvic acid	*T. delbrueckii*	+	Belda et al., 2015
	T. delbrueckii + *S. c.*	+	Belda et al., 2015
	C. stellata + *S. c. L. thermotolerans* + *S. c.*	+	Soden et al., 2000; Jolly et al., 2006; Belda et al., 2015
Glycerol	*T. delbrueckii* + *S. c.*	+	Benito et al., 2016
	C. stellata + *S. c. L. thermotolerans* + *S. c.*	+	Soden et al., 2000; Jolly et al., 2006; Benito et al., 2016
	S. bacillaris + *S. c.*	+	Englezos et al., 2016b
Mannoproteins	*M. pulcherrima* + *S. c.*	+	Belda et al., 2016
	T. delbrueckii + *S. c.*	+	González-Royo et al., 2015; Belda et al., 2016

**S. c. corresponds to Saccharomyces cerevisiae.*

acid and β-phenylethanol (Caridi and Corte, 1997). More recent studies agreed with the inhibition effect of succinic acid (Son et al., 2009), and not with its role as MLF extender.

Citric Acid

Even though citric acid is considered as inhibitor of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982), citric acid can be catabolized by LAB (Liu, 2002). This metabolic activity is found in some *O. oeni* strains as response to acidity or ethanol stress (Olguín et al., 2009). Due to the consumption of citric acid, diacetyl is produced (Swiegers et al., 2005). It is usually desirable to have strains which can consume citric acid due to the organoleptic complexity that is achieved (Lonvaud-Funel, 1999). In this way, a high concentration of diacetyl is reported as undesirable (Davis et al., 1985; Bartowsky and Henschke, 2004). Moreover, due to the citric acid metabolism, *O. oeni* increases the volatile acidity (Lonvaud-Funel, 1999; Liu, 2002). Even thought, citric acid increases the transmembrane gradient which generate energy in terms of proton-motive force for *O. oeni* (Liu Y. et al., 2016).

Anyway, since citric acid concentration is usually not very high, acetic acid does not increase very much. Citric acid production by yeast is highly species and strain dependent (Fleet, 2008). On the top of that, mixed fermentations with different non-*Saccharomyces* species exhibit particular citric acid production (Jussier et al., 2006; Giaramida et al., 2013; Izquierdo Cañas et al., 2014). For the moment the only mixed fermentation that clearly increased citric acid concentration is with *S. bacillaris* (Giaramida et al., 2013).

Pyruvic Acid

Pyruvic acid is an intermediary produced by yeast during the AF. This compound can improve MLF performance by *O. oeni*. It acts as external electron acceptor, facilitating the regeneration of NAD^+ (Maicas et al., 2002). It can also promote diacetyl production (Mink et al., 2015). Related to increasing the concentration of this compound, Belda et al. (2015) reported higher production of pyruvic acid when *T. delbrueckii* was used as sole or mixed culture starter with *S. cerevisiae*. Benito et al. (2016) reported similar results using *L. thermotolerans*.

Glycerol

The production of glycerol is directly related with the activity of yeasts by the glyceropyruvic fermentation pathway (Ciani and Maccarelli, 1998). Glycerol can be assimilated and degraded by some spoiling *Lactobacillus* in wine (Liu, 2002). On the contrary, there is no literature that reports this behavior when it comes to *O. oeni*. It is unclear how can affect glycerol

to *O. oeni*, since it does not assimilate it, neither degrade it. Usually, non-*Saccharomyces* yeasts exhibit higher metabolic activity of this pathway (Ciani and Maccarelli, 1998; Jolly et al., 2006, 2014). Specifically, *T. delbrueckii* (Belda et al., 2015) and *C. stellata* (Soden et al., 2000; Jolly et al., 2006) have been reported as big glycerol and pyruvic acid producers as result of their high glyceropyruvic fermentation activity. Also, mixed fermentations with *S. bacillaris* and *L. thermotolerans* exhibit higher production of glycerol in regards to a conventional *S. cerevisiae* fermentation (Benito et al., 2016; Englezos et al., 2016b).

Compounds Derived of Yeast Autolysis

One of the most known positive effects upon MLF performance is its development in presence of yeast lees (Guilloux-Benatier et al., 1995). It has been reported that the inhibitory interactions between yeasts and LAB is counteracted by the presence of yeast lees, and even more, the positive interactions are enlarged (Patynowski et al., 2002). During aging, yeasts undergo an autolytic process that result in the release of different compounds. Nitrogenated compounds, such as amino acids, peptides and proteins, are mainly released as result of yeast autolysis (Guilloux-Benatier et al., 1995; Martínez-Rodriguez et al., 2001). The release of such compounds can help to enrich the previously exhausted medium by yeasts (Costello et al., 2003), stimulating the growth of LAB and MLF performance (Guilloux-Benatier et al., 1995; Diez et al., 2010).

Other molecules like glucans and mannoproteins are also released due to this mentioned process and can stimulate LAB growth (Diez et al., 2010). These authors realized that the presence of mannoproteins only exhibited its positive effect on LAB growth when ethanol was present. *O. oeni* can catabolize these mannoproteins and release mannose, which can be substrate of the phosphotransferase system that helps the adaptation of *O. oeni* to the medium (Jamal et al., 2013). Besides this, the impact of the mannoproteins upon LAB was yeast-LAB strain dependent. Recently, it has been reported that some non-*Saccharomyces* strains belonging to *M. pulcherrima* and *T. delbrueckii* release more mannoproteins than *S. cerevisiae* (Belda et al., 2016). Moreover, these molecules could help hijack MCFA present in wine, stimulating LAB growth (Guilloux-Benatier et al., 1995). It has been also been reported that during AF those cited macromolecules are released, depending in the initial colloidal concentration (Guilloux-Benatier et al., 1995). Still, the same study states that the amount of macromolecules released during yeast growth is insignificant in regards to yeast autolysis.

Apart from the mentioned compounds, there are more released compounds during yeast autolysis, such as vitamins, nucleotides and long chain fatty acids, which could be also stimulatory to LAB (Alexandre et al., 2004). Unfortunately, there is no literature currently available about the possible effect of these compounds.

Other Compounds

In regards to the possible incompatibility between oenological yeasts and LAB, apart from metabolite compounds, the production of antimicrobial proteinaceous compounds by some *S. cerevisiae* strains has been reported. Dick et al. (1992) firstly studied these compounds. They discovered two cationic proteins which were effective against LAB. More recently, another inhibitory protein fraction produced by *S. cerevisiae* CCMI 885 and active against LAB was identified (Branco et al., 2014). In this work, an exhaustive characterization was performed, which resulted in the identification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein fragments. This newly identified antimicrobial peptides with 2–10 kDa size agreed with previously reported antimicrobial peptides (Comitini et al., 2005; Osborne and Edwards, 2007).

There are no studies about these compounds produced by non-*Saccharomyces* yeasts, but some species could present such antimicrobial compounds, like *M. pulcherrima* that produce pulcherrimic acid (Oro et al., 2014), active against other yeasts.

FUTURE PERSPECTIVES

The increasing number of non-*Saccharomyces* species described as beneficial in winemaking demands further investigation of their metabolism. Many factors can influence the effect of non-*Saccharomyces* on wine composition. Besides the yeast species and strain characteristics, the time and the ratio of inoculation, with respect to *S. cerevisiae*, may alter notably the global effect on wine of the use of non-*Saccharomyces*. All these variables may also affect the development of *O. oeni* and MLF. Future research should contribute to a better knowledge of metabolic traits of a wider number of non-*Saccharomyces* strains and their influence on *O. oeni* performance. Among other possible approaches, metabolomics may be a powerful tool to elucidate how the new winemaking scenario of combined yeasts may change MLF evolution.

AUTHOR CONTRIBUTIONS

All authors conceived, drafted the manuscript, and approved the final version of the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annex III

Effect of the inoculation strategy of non-*Saccharomyces* yeasts on wine malolactic fermentation

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Effect of the inoculation strategy of non-*Saccharomyces* yeasts on wine malolactic fermentation

Alba Martín-García, Aitor Balmaseda, Albert Bordons* and Cristina Reguant

Grup de Biotecnologia Enològica, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, c/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

*corresponding author: albert.bordons@urv.cat

ABSTRACT

Interest in some non-*Saccharomyces* yeasts has increased recently, because they have been associated with an improvement in wine quality. Nevertheless, little attention has been paid to the effect that the use of these yeasts may have on malolactic fermentation (MLF). In this study, the strains *Torulaspora delbrueckii* Biodiva and *Metschnikowia pulcherrima* Flavia were evaluated by co-inoculation and sequential fermentation with *S. cerevisiae* QA23. A fermentation with *S. cerevisiae* as a single starter was also performed as a control, then MLF was performed inoculating *Oenococcus oeni* PSU-1 in all wines. Finally, the wines obtained after alcoholic fermentation and MLF were characterised. The results of the coinoculated fermentations were similar to those of the *S. cerevisiae* control fermentations. Nevertheless, significant differences were observed in sequential fermentations in terms of lower content of acetic, L-malic and succinic acids. These differences were particularly noticeable in fermentations carried out with *T. delbrueckii*.

KEYWORDS

malolactic fermentation, *Metschnikowia pulcherrima*, non-*Saccharomyces*, *Oenococcus oeni*, *Torulaspora delbrueckii*, wine

INTRODUCTION

There has been an increasing interest in inoculating grape musts with non-*Saccharomyces* yeasts to complement the traditional usage of *Saccharomyces cerevisiae* as a sole starter, as they improve product quality and complexity (Ciani *et al.*, 2010; Comitini *et al.*, 2011; Contreras *et al.*, 2014; Jolly *et al.*, 2003, 2014; Whitener *et al.*, 2015; Zott *et al.*, 2011). These other yeast species have little or moderate fermentation power and *S. cerevisiae* must be inoculated to finish the alcoholic fermentations (AF) (Benito *et al.*, 2015). Thus, a new trend in winemaking uses mixed starter cultures of non-*Saccharomyces* and *S. cerevisiae* (Belda *et al.*, 2015; Ciani *et al.*, 2010) or a sequential inoculation of *S. cerevisiae* after non-*Saccharomyces* (Contreras *et al.*, 2014; González-Royo *et al.*, 2015). Among the different species of non-*Saccharomyces*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* show the most promising results for global wine quality, such as low production of volatile acidity (Renault *et al.*, 2009) or a notable mannoprotein release ability, which increases the mouthfeel properties of wine (Belda *et al.*, 2016).

Malolactic fermentation (MLF) consists of the decarboxylation of L-malic acid to L-lactic acid by the lactic acid bacteria (LAB), mainly *Oenococcus oeni*. In addition to decreasing wine acidity, MLF induces other changes such as microbiological stability or organoleptic improvement (Bartowsky, 2005).

The performance of MLF by LAB is affected by the intrinsic properties of wine, which are mostly determined by yeasts (Balmaseda *et al.*, 2018). The effects of yeasts on MLF can be either inhibitory, for example the production of ethanol or the nutrient exhaustion (Arnink and Henick-Kling, 2005), or stimulating, such as the production of citric and pyruvic acids (Liu *et al.*, 2016). These effects depend on the concentration of the compounds in wine, which, in turn, depends on species and strains (Balmaseda *et al.*, 2018).

The aim of this study was to determine the effect of the species of non-*Saccharomyces* with interesting oenological traits (*T. delbrueckii* and *M. pulcherrima*) on the MLF, by evaluating and comparing the inoculation strategies of co-inoculation (non-*Saccharomyces* and *S. cerevisiae*), or their sequential inoculation at different times.

MATERIALS AND METHODS

1. Microorganisms and inocula

The yeast strains used were *T. delbrueckii* Biodiva (Td), *M. pulcherrima* Flavia (Mp) and *S. cerevisiae* Lalvin-QA23 (Sc), all from Lallemand Inc. (Montréal, Canada). Strain *O. oeni* PSU-1 (ATCC BAA-331) was used for the MLF. Yeasts were maintained on YPD plates (2 % glucose, 2 % bacto-peptone, 1 % yeast extract, 2% agar, w/v) and bacteria on MRSmf (Margalef-Català *et al.*, 2017) plates, and all were stored at 4 °C. To obtain the inocula, a colony was picked from the plates and grown in liquid media YPD at 28 °C (yeasts) and MRSmf at 27 °C in a 10 % CO_2 atmosphere (*O. oeni*). Then, aliquots of 400 µL of these preinocula were inoculated in 40 mL of the same fresh liquid media.

2. Experimental fermentations

Fermentations were performed in 500 mL flasks containing 400 mL of sterile must, prepared using white grape concentrated must (65.4 ° Brix; Mostos Españoles S.A., Tomelloso, Spain) and sterile MilliQ purified water to obtain a sugar concentration of 200 ± 10 g/L.

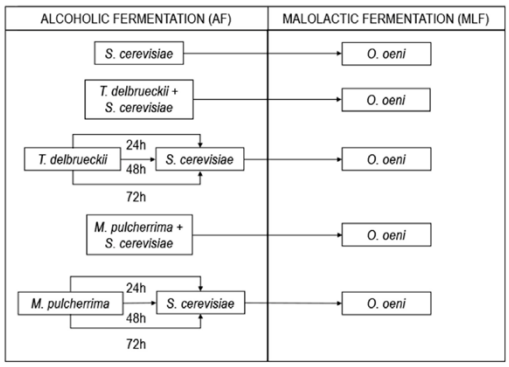

FIGURE 1. Diagram of the experimental fermentations. Each one was carried out by triplicate.

Alcoholic fermentations (AF) were carried out with two non-*Saccharomyces* strains and the inoculation of *S. cerevisiae* was performed in different time regimes: co-inoculation (Td-Sc; Mp-Sc), after 24 h (Td.24 h; Mp.24 h), 48 h (Td.48 h; Mp.48 h) and 72 h (Td.72 h; Mp.72 h). A control fermentation was also performed, with *S. cerevisiae* as the sole starter (Sc) (Figure 1).

Each yeast was inoculated for a population of 10^6 cells/mL. All fermentations were carried out in triplicate. Samples were taken every 24 h to monitor the evolution of sugar consumption and yeast population. YPD agar was used to calculate the total number of yeast cells, and lysine agar medium (Oxoid LTD, England) was used to quantify the non-*Saccharomyces* (Wang *et al.*, 2016) after incubation at 28 ºC for 48 h. AF was considered to have finished when the sugar concentration was below 1 g/L. To eliminate all yeasts, the resulting wines were filtered (MF-Millipore™ 0.45 µm, Merck Millipore, Madrid, Spain).

Next, each wine (100 mL) was inoculated with *O. oeni* for a population of 2×10^7 cells/mL. These fermentations were also carried out in triplicate. Samples were taken every 24 h to monitor the evolution of L-malic acid consumption and the bacterial population. Samples were plated on MRSmf and incubated at 27 °C in a 10 % CO_2 atmosphere for 7 days. MLF was considered to have finished when L-malic acid was below 0.05 g/L

3. Wine characterisation

After AF, ethanol content was determined by enzymatic assay (R-Biopharm AG, Darmstadt, Germany). On completion of AF and MLF, pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain) and various compounds (acetic acid, citric acid, L-lactic acid, L-malic acid, ammonium, α-NH_2, succinic acid, glycerol, glucose+fructose, total and free SO_2) were analysed with the multianalyser Miura One (TDI SL, Gavà, Spain).

4. Statistical analysis

Statistical software XLSTAT version 2018.4.51298 (Addinsoft, Paris, France) was used. The data obtained was submitted to one-way ANOVA with subsequent analysis using the Tukey test, with a confidence interval of 95 % and significant results with a p-value of ≤ 0.05. Principal component analysis (PCA) was also performed to determine differences between the wines.

RESULTS AND DISCUSSION

1. Alcoholic fermentation

Control fermentation with only *S. cerevisiae* was completed in 10 days, with a sugar consumption rate of 31.25 g/L·day (Table 1). This was the fastest fermentation performed because, unlike coinoculated and sequential fermentations, there was neither synergy nor competition for the substrate between yeasts. Coinoculated Td-Sc and Mp-Sc fermentations was completed in 11 days with a lower consumption rate (Table 1).

Table 1 shows that sequential fermentations of *T. delbrueckii* (Td.24 h, 48 h, 72 h) took longer than the control fermentations, largely because the final stages were slower (Figure 2 left), while fermentations of *M. pulcherrima* had slow early stages but finished at the same time as the control. In fact, sugar consumption was not significant until *S. cerevisiae* was inoculated (Figure 2, right). Nevertheless, during the initial days the non-*Saccharomyces* populations were stable and did not decrease until the *S. cerevisiae* inoculation (data not shown).

TABLE 1. Alcoholic (AF) and malolactic (MLF) fermentations duration and speed.

	AF duration (d)	AF speed* (g/L·d)	MLF duration (d)	MLF speed* (g/L·d)
Sc	10 ± 3^{a}	31.25 ± 2.04^{c}	3 ± 0^{a}	0.56 ± 0.11^{a}
Td-Sc	11 ± 1^{ab}	29.24 ± 1.53^{c}	7 ± 1^{d}	0.23 ± 0.02^{d}
Td.24 h	20 ± 0^{e}	22.05 ± 0.31^{b}	5 ± 0^{abc}	0.29 ± 0.01^{cd}
Td.48 h	25 ± 1^{f}	16.80 ± 1.07^{a}	4 ± 0^{ab}	0.48 ± 0.02^{ab}
Td.72 h	18 ± 0^{de}	19.29 ± 1.29^{ab}	4 ± 0^{ab}	0.53 ± 0.02^{a}
Mp-Sc	11 ± 0^{ab}	37.00 ± 2.08^{de}	4 ± 0^{ab}	0.40 ± 0.02^{bc}
Mp.24 h	14 ± 0^{cd}	30.40 ± 0.60^{c}	5 ± 2^{bcd}	0.33 ± 0.03^{cd}
Mp.48 h	14 ± 1^{abc}	37.80 ± 0.83^{e}	6 ± 1^{bcd}	0.35 ± 0.01^{cd}
Mp.72 h	14 ± 0^{bc}	33.15 ± 1.65^{cd}	7 ± 2^{cd}	0.32 ± 0.04^{cd}

Values shown are the means of triplicates ± SD. *Calculation based on consumption speed of sugar (AF) and L-malic acid (MLF) considering the period of exponential decrease of these compounds. $^{a-d}$, vales are significantly different at $p \leq 0.05$, according to a Tukey post-hoc comparison test.

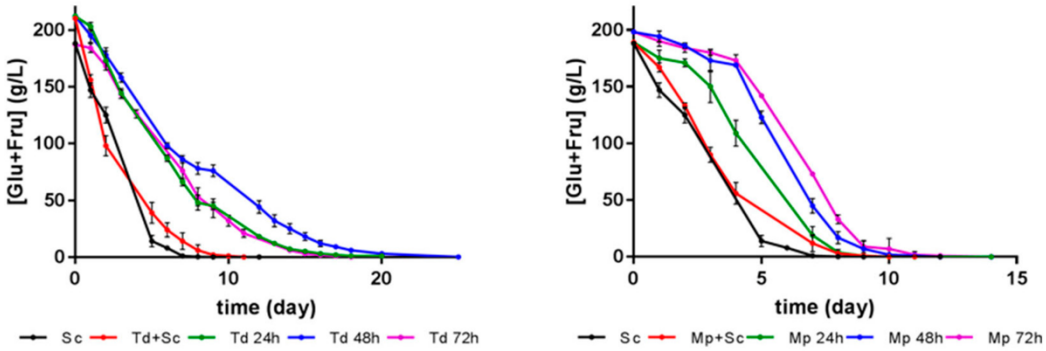

FIGURE 2. Evolution of alcoholic fermentation through sugar consumption by yeasts.
Left: *T. delbrueckii* fermentations (Td-Sc; Td.24 h; Td.48 h; Td.72 h) and control (Sc). Right: *M. pulcherrima* fermentations (Mp-Sc; Mp.24 h; Mp.48 h; Mp.72 h) and control (Sc).

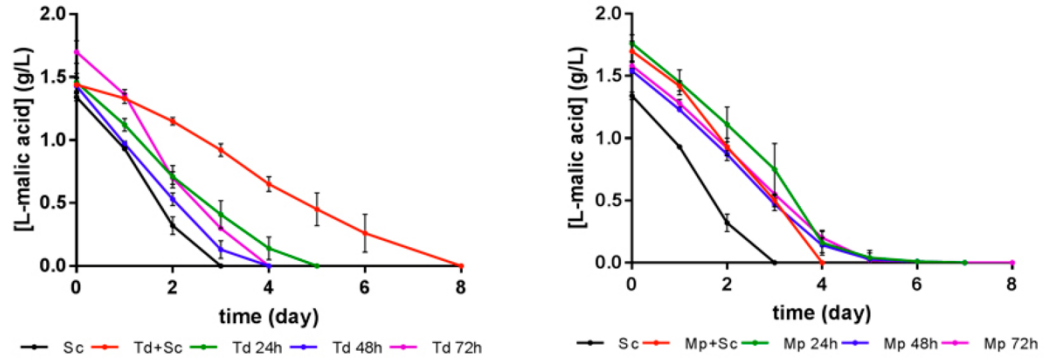

FIGURE 3. Evolution of malolactic fermentation after AF by monitoring the L-malic acid consumption by *O. oeni* PSU-1.
Left: wines fermented with *T. delbrueckii* (Td-Sc; Td.24 h; Td.48 h; Td.72 h) and control (Sc). Right: wines fermented with *M. pulcherrima* (Mp-Sc; Mp.24 h; Mp.48 h; Mp.72 h) and control (Sc).

2. Malolactic fermentation

No significant differences were observed in MLF between Sc and non-*Saccharomyces* wines (considering the exponential decrease in L-malic acid), except for Mp 72 h, which was slower (Table 1 and Figure 3). Nonetheless, there were significant differences in terms of MLF duration between non-*Saccharomyces* species. MLF was slower in wines produced from Mp sequential fermentations and Td-Sc.

3. Changes in wine composition

As expected, the inoculation strategy had an impact on wine composition after AF (Table 2). The most relevant changes were observed in sequential inoculation, which were related to the longer persistence of non-*Saccharomyces* populations, as introduced above. Td fermentations showed no significant differences in ethanol content with respect to the control. Although *T. delbrueckii* has been reported to produce less ethanol than *S. cerevisiae* (Contreras *et al.*, 2014), several other authors have found almost no difference (Belda *et al.*, 2015) and thus it may depend on the strain and the conditions (Benito, 2018).

On the contrary, Mp sequential fermentations were found to have significantly lower ethanol content, especially in Mp.24 h. The production of glycerol was observed to be similar in Sc and Td wines. However, Mp fermentations presented a significantly higher content at the end of AF, as has been observed elsewhere (Contreras *et al.*, 2014). This may be due to the higher capacity of non-*Saccharomyces* yeasts (Mp) to use the glycopyruvic pathway instead of the usual pyruvate-to-ethanol pathway (Jolly *et al.*, 2014).

TABLE 2. Characterisation of initial must and wines after alcoholic (AF) and malolactic (MLF) fermentations.

	Sugar (g/L)	L-malic acid (g/L)	Citric acid (g/L)	α-NH_2 (mg/L)	Ammonium (mg/L)	pH
Initial must	198.11 ± 10.72	2.14 ± 0.08	0.25 ± 0.05	153.90 ± 11.21	70.22 ± 4.44	3.94 ± 0.01

	Sugar (g/L)	Ethanol	L-malic acid (g/L)	pH		Citric acid (g/L)		Acetic acid (g/L)	
	AF	(% (v/v))	AF	AF	MLF	AF	MLF	AF	MLF
Sc	0.11 ± 0.09	11.67 ± 0.29^{a}	1.36 ± 0.03^{d}	3.45 ± 0.03^{e}	3.86 ± 0.02^{bc}	0.22 ± 0.02	0.01 ± 0.01^{cd}	0.23 ± 0.04^{bc}	0.41 ± 0.03^{ab}
Td-Sc	0.23 ± 0.21	11.12 ± 0.28^{ab}	1.46 ± 0.06^{cd}	3.56 ± 0.09^{de}	3.74 ± 0.00^{de}	0.23 ± 0.03	0.17 ± 0.03^{a}	0.18 ± 0.02^{cd}	0.28 ± 0.02^{cd}
Td.24 h	0.63 ± 0.23	11.48 ± 0.08^{a}	1.46 ± 0.07^{cd}	3.85 ± 0.05^{ab}	4.20 ± 0.02^{a}	0.28 ± 0.01	0.09 ± 0.01^{b}	0.12 ± 0.02^{d}	0.22 ± 0.01^{d}
Td.48 h	nd	11.51 ± 0.11^{a}	1.43 ± 0.06^{cd}	3.93 ± 0.04^{a}	4.23 ± 0.00^{a}	0.24 ± 0.01	nd^{d}	0.12 ± 0.03^{d}	0.25 ± 0.03^{d}
Td.72 h	0.10 ± 0.09	11.16 ± 0.28^{a}	1.70 ± 0.08^{ab}	3.81 ± 0.01^{abc}	4.03 ± 0.02^{b}	0.22 ± 0.02	nd^{d}	0.10 ± 0.01^{d}	0.29 ± 0.01^{bcd}
Mp-Sc	0.16 ± 0.05	11.64 ± 0.10^{a}	1.70 ± 0.08^{ab}	3.49 ± 0.04^{de}	3.93 ± 0.01^{bc}	0.22 ± 0.02	0.01 ± 0.01^{cd}	0.35 ± 0.02^{a}	0.49 ± 0.02^{a}
Mp.24 h	0.23 ± 0.20	7.88 ± 0.21^{d}	1.76 ± 0.07^{a}	3.46 ± 0.00^{de}	3.97 ± 0.09^{bc}	0.21 ± 0.03	nd^{d}	0.31 ± 0.04^{bc}	0.35 ± 0.06^{abc}
Mp.48 h	0.13 ± 0.09	9.96 ± 0.16^{c}	1.54 ± 0.02^{c}	3.73 ± 0.07^{bc}	3.88 ± 0.13^{bc}	0.23 ± 0.01	0.05 ± 0.04^{bc}	0.29 ± 0.03^{ab}	0.35 ± 0.01^{abc}
Mp.72 h	0.25 ± 0.20	10.35 ± 0.18^{bc}	1.58 ± 0.02^{bc}	3.65 ± 0.03^{cd}	3.81 ± 0.08^{c}	0.29 ± 0.04	0.03 ± 0.01^{cd}	0.19 ± 0.05^{cd}	0.30 ± 0.05^{bcd}

	Glycerol (g/L)		Succinic acid (mg/L)	α-NH_2 (mg/L)		Ammonium (mg/L)		Total sulfite (mg/L)		Free sulfite (mg/L)
	AF	MLF	AF	AF	MLF	AF	MLF	AF	MLF	AF
Sc	5.06 ± 0.20^{d}	5.80 ± 0.44	331.94 ± 2.98^{a}	32.54 ± 1.24^{b}	32.23 ± 2.48^{b}	3.00 ± 0.71	nd	45.50 ± 0.71^{ab}	13.67 ± 2.31^{ab}	4.00 ± 1.00^{a}
Td-Sc	5.56 ± 0.38^{cd}	6.17 ± 0.19	323.82 ± 3.66^{ab}	36.89 ± 2.34^{b}	41.79 ± 0.09^{b}	0.67 ± 0.57	3.00 ± 0.00	39.50 ± 0.71^{abc}	2.00 ± 1.41^{d}	2.00 ± 1.73^{ab}
Td.24 h	6.28 ± 0.09^{cd}	6.52 ± 0.02	311.53 ± 3.96^{bc}	65.73 ± 5.54^{a}	61.57 ± 2.93^{a}	2.50 ± 0.71	3.00 ± 1.00	26.67 ± 3.05^{cde}	4.67 ± 3.79^{bcd}	3.33 ± 0.58^{ab}
Td.48 h	5.74 ± 0.27^{cd}	5.92 ± 0.07	309.66 ± 1.81^{c}	73.06 ± 5.38^{a}	66.37 ± 2.71^{a}	2.50 ± 0.71	5.50 ± 0.71	16.00 ± 2.82^{e}	5.00 ± 1.41^{cd}	1.67 ± 0.58^{ab}
Td.72 h	5.87 ± 0.27^{cd}	4.59 ± 0.01	314.20 ± 1.15^{bc}	63.80 ± 3.52^{a}	60.47 ± 0.74^{a}	1.00 ± 0.00	3.50 ± 0.71	18.00 ± 0.00^{de}	7.00 ± 0.00^{bcd}	1.50 ± 0.71^{b}
Mp-Sc	6.19 ± 0.21^{cd}	4.97 ± 0.01	320.28 ± 1.28^{bc}	26.98 ± 4.02^{b}	28.90 ± 1.24^{b}	3.50 ± 1.41	2.00 ± 0.00	45.67 ± 0.00^{a}	18.00 ± 2.83^{a}	3.67 ± 0.57^{a}
Mp.24 h	6.45 ± 0.14^{bc}	5.29 ± 0.18	320.29 ± 7.01^{bc}	20.26 ± 2.00^{b}	20.00 ± 3.70^{b}	2.00 ± 1.00	2.00 ± 1.00	28.00 ± 4.24^{abc}	15.33 ± 3.79^{a}	2.33 ± 0.58^{ab}
Mp.48 h	7.27 ± 0.21^{b}	7.05 ± 0.16	315.53 ± 2.60^{bc}	39.07 ± 8.30^{b}	38.77 ± 3.03^{b}	4.00 ± 0.00	3.50 ± 0.71	33.00 ± 2.00^{abc}	20.67 ± 3.79^{a}	2.33 ± 0.58^{ab}
Mp.72 h	8.01 ± 0.25^{a}	7.50 ± 0.65	309.42 ± 1.69^{c}	28.32 ± 5.33^{b}	19.97 ± 3.87^{b}	7.00 ± 0.00	3.33 ± 1.53	29.67 ± 2.89^{bcd}	13.33 ± 1.53^{abc}	2.33 ± 0.58^{ab}

$^{a-d}$, values are significantly different at $p \leq 0.05$, according to a Tukey post-hoc comparison test, values without superscript letters did not show significant differences.

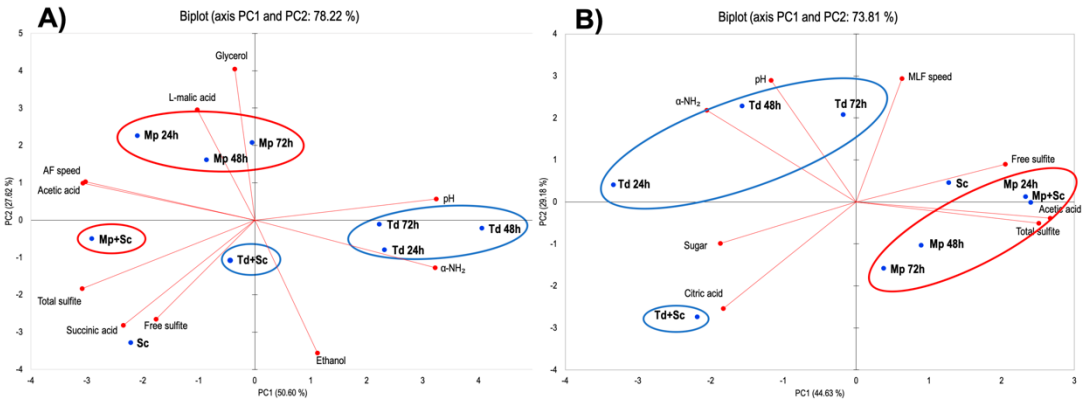

FIGURE 4. Principal component analysis (PCA) biplot of wines obtained at the end of (A) alcoholic fermentation and (B) malolactic fermentation.
The values shown are the mean of triplicates

The pH was significantly higher in Td wines with sequential inoculation, and was closer to initial must pH. In addition, Mp.48 h and Mp.72 h showed significantly higher pH than control Sc. A higher pH can be an attenuating factor for the inhibitory effect of ethanol on *O. oeni*, but MLF was not shorter in Td sequential wines. Despite this, under non-sterile cellar conditions, a pH close to 4 or higher may promote the development of other LAB, such as *Pediococcus* spp. and threaten wine quality (Wade *et al.*, 2019).

Both *S. cerevisiae* and non-*Saccharomyces* consumed some L-malic acid during AF (Table 2). In Mp sequential fermentations, consumption was higher when it took longer to inoculate Sc, although all non-*Saccharomyces* consumed less L-malic acid than Sc. Nevertheless, high values of L-malic acid tend to ensure a good MLF. L-lactic acid production depended on L-malic acid consumption (data not shown).

Citric acid content did not vary during AF. No differences were found in its metabolisation by *O. oeni*, except for Td-Sc and Td.24 h, in which it was not completely consumed, unlike other fermentations. In Td fermentations, MLF was slower when *O. oeni* did not totally consume the citric acid.

Acetic acid concentration after AF was up to 60 % lower in Td sequential AF wines. It has been observed elsewhere that some non-*Saccharomyces* can decrease acetic acid concentration (Chen *et al.*, 2018). Data obtained from Mp wines appeared to be similar to the control data, although Mp-Sc was higher, probably due to the early imposition of Sc. After MLF, as expected, the acetic acid concentration was higher due to citrate consumption.

In agreement with other studies, succinic acid production decreased in non-*Saccharomyces* AF (Contreras *et al.*, 2014). These differences were most noticeable in Mp fermentations, in which succinic acid decreased by up to 10 % more than Sc fermentation. Succinic acid can act as a competitive inhibitor of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982), which has a negative effect on MLF, although this inhibition has not been observed in the present study.

Td sequential fermentations consumed the least α-NH_2 - the free alpha-amino nitrogen that is equivalent to available amino acids (Table 2). The coexistence of the two yeast populations may have resulted in higher nitrogen consumption. This data is in agreement with other studies reporting competition for nitrogen sources between yeasts (Gobert *et al.*, 2017). No significant differences in ammonium consumption by yeasts were observed here.

One of the main products of the antagonistic interactions between yeasts and *O. oeni* is SO_2 (Nehme *et al.*, 2008). Some non-*Saccharomyces* strains can produce significant amounts of SO_2 (Wells and Osborne, 2011). In this study, differences were found between Td wines and the others. Sequential Td wines showed that total SO_2 production was lower and the content of free SO_2 was similar. The lack of any difference between Mp and Sc wines may be the consequence of an

early imposition of the latter. The values of total SO_2 were clearly lower after MLF than before it (Table 2). This could be explained by the known reduction of bound SO_2 levels due to degradation of acetaldehyde and other binding compounds by *O. oeni* during MLF (Davis *et al.*, 1985; Jackowetz and Mira de Orduña, 2012).

Considering the variables studied, the PCA (Figure 4) confirmed the differences between yeast species. It can be observed that in wines after AF (Figure 4 A), Td sequential fermentations are clustered in one group and Mp sequential fermentations are grouped in another. The first group consumes less α-NH_2 and has a higher pH, while the second has a lower ethanol but higher glycerol content. This shows that there are similarities between wines fermented with the same non-*Saccharomyces* species, regardless of the time of inoculation with *S. cerevisiae*.

After MLF (Figure 4 B), the clusters were maintained with slight differences. Mp-Sc wine is clustered with the other sequential fermentations of Mp. In addition, all wines are closer in the PCA, indicating a homogenisation of wines after MLF due to the metabolism of the *O. oeni* strain used.

CONCLUSIONS

This study shows that the impact of non-*Saccharomyces* was greater on sequential AF than on coinoculated AF. Differences were observed between *T. delbrueckii* and *M. pulcherrima*. When *T. delbrueckii* was used, it had a positive effect on *O. oeni* and MLF due to lower acidity, succinic acid and SO_2, even though MLF was slightly slower than in *S. cerevisiae* wines. *M. pulcherrima* decreased ethanol content during AF, which minimised its negative effect on *O. oeni*, yet MLF was slower than in control wines. Thus, other compounds must have a negative effect on *O. oeni*.

Further research is required for a better understanding of the impact of non-*Saccharomyces* on MLF

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